IDENTIFICATION OF PROTEIN PARTNERS AND CHARACTERIZATION OF FUNCTIONAL DOMAINS OF PININ

By

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Pinin is a 140 KD phosphoprotein found at the desmosome-intermediate filament complex and within the nucleus of epithelial cells. Epithelial adhesion assembly assays indicated that the location of pinin was dynamic. Furthermore, the presence of pinin was correlated with increased organization and stabilization of desmosome-intermediate filament complex. Transfection of the cDNA coding for pinin into transformed cells demonstrated that pinin played a role in maintaining/conferring the epithelial polarity as well as modulating epithelial growth quality (loss of anchorage-independent growth of tumor cells). Pinin's affect on tumor cell growth, the observed dysregulation of the expression of pinin in a subset of cancer cells, and the mapping of pinin to a known tumor suppressor locus indicate a possible tumor suppressive function of pinin. However, the precise molecular mechanism pinin employs to achieve these functions in

various cellular events are as of yet unclear. As a step toward elucidating the precise molecular mechanisms of pinin's activity, we sought to identify proteins that interact with pinin. We employed the yeast two-hybrid system to identify candidate pinin binding partners. Both the amino and the carboxyl portions of pinin were used as bait in two-hybrid screens on a human fetal kidney cDNA library. The amino portion of pinin was shown to bind to a group of adhesion-cytoskeleton-related proteins, including keratin, exo70, syntaxin 4, as well as novel proteins such as periplakin-like and trichohyalin-like proteins. In contrast, the carboxyl portion of pinin exhibited binding to a subset of nuclear proteins, such as SRp75, SRm300 as well as a novel SR protein. Furthermore, truncation and site-directed mutagenesis were employed to more precisely define the respective binding sites. The data generated from this study are consistent with our previous morphological observations of pinin's dual location at the desmosome-IF complex and in the nucleus. The identification of proteins interacting with pinin provides important fundamental information pertaining to possible cellular events in which pinin may be involved. The characterization of specific domains within pinin and within the target proteins will afford us the opportunity to manipulate specific pinin interactions and in turn dissect the molecular mechanism of pinin's function. This work, in combination with future studies, will greatly contribute to our current understanding of cell-cell adhesion as well as adhesion related intracellular events.

CHAPTER 1
BACKGROUND

Introduction

Epithelial cell adhesion, achieved by both junctional and non-junctional adhesions, plays significant roles in embryogenesis, tissue morphogenesis, epitheliogenesis, as well as in the regulation of cell migration and proliferation. While non-junctional adhesions may be essential for the initial establishment of cell-cell contact, specialized cellular junctions are structural and functional units of cell adhesions and can be classified as two groups. Cell-matrix junctions such as hemidesmosomes locate at the basal membrane of epithelial cells, participating in anchoring epithelia on solid substrate and receiving signals from the extracellular matrix (ECM). Cell-cell junctions, such as tight-junctions, adherens junctions, desmosomes, reside at the lateral cell surface, mediating cell-cell adhesion that allows for epithelia functioning as a whole. Most of these junctions are multi-protein complexes composed of transmembrane proteins as well as cytoplasmic plaque/peripheral proteins assembled via protein-protein interactions. The extracellular domains of the transmembrane proteins serve to connect to either ECM or the neighboring cells while the cytoplasmic domains of the transmembrane proteins interact with either peripheral proteins or cytoskeleton such as microfilaments and intermediate filaments, thus structurally assemble the epithelia together. Overall, cell junctions serve as the sites of adhesion as well as the sites of

reinforcements for structural/functional integrity of the epithelia. In addition, cell adhesion junction molecules may respond to extracellular stimulation, disassemble from the junction and/or alter their protein contacts, conferring signaling functions to the junctions.

Our lab has been interested in cell adhesion and focused on investigations of a desmosome-IF associated and nucleus-localized protein, pinin (Brandner et al., 1997; Brandner et al., 1998; Ouyang, 1999; Ouyang and Sugrue, 1992; Ouyang and Sugrue, 1996). Pinin was first identified in our lab using a mAb generated against insoluble cellular preparation of MDCK cells (Ouyang and Sugrue, 1992). Previous studies have achieved remarkable progress in revealing the nature of pinin and the involvement of pinin in cell-cell adhesion. However, as in most scientific discovery processes, the more we have learned about pinin, the more questions arise. At present, the function of pinin is thought to be far beyond the traditional theme of desmosomes and now pinin is considered as a multi-functional protein with dual locations in the cell. However, the specific functions and molecular mechanisms involved by pinin remain largely unknown.

In general, most proteins in cells associate with particular protein complex for reasons probably but not necessarily related to their functions. Identifying proteins capable of interacting with pinin using library-screening methodology is promising to reveal the possibilities of molecular connections pinin may be involved in, which in turn will enable us to further pursue the biological functions of pinin and to view pinin functions as a whole. In this study, a two-hybrid system was employed to identify potential protein partners of pinin. Intriguingly, groups of proteins including intermediate filament protein keratins, potential desmosomal proteins, nuclear RS

domain-containing proteins and several other interesting proteins were identified. I will present the two-hybrid screening data in chapter two, the detailed study on pinin-keratin relationship in chapter three, and the RS domain-containing protein-pinin relationship in chapter four. Therefore, in this first chapter, I would like to introduce some related background information on pinin, intermediate filaments and related proteins, desmosomes, and nuclear matrix and nuclear substructures.

Pinin

Sequence analysis of pinin cDNA and the implications. Human pinin gene has been located to chromosome 14 by fluorescence in situ hybridization (FISH). Northern Blot revealed the existence of pinin isoforms in several tissues. However, so far identified pinin cDNA in canine, bovine, human (Ouyang and Sugrue, 1996), as well as in Xenopus (Brandner et al., 1997) exhibit high homology with each other, indicating the conservation of this gene during the evolution. The conceptual translation product of the cDNA provided limited indications on possible functions of pinin. Nevertheless, several distinctive domains were recognized (Fig. 1.1).

At the amino end of pinin sequence, there are four and a half heptad repeats predicted to form coiled-coil structure by computer programs COILs (Lupas, 1996b) and PAIRCOIL (Berger et al., 1995). Heptad repeats is a stretch of sequence characteristics of having hydrophobic amino acid at the first and the fourth of every seven residues. It is estimated that 3% of the peptide sequences in the database are potential coiled-coil motifs (Lupas et al., 1991). Proteins with known structure containing coiled-coil motifs include IF proteins, cytoskeleton associated protein such as tropomyosin, a subset of transcription

factors that contain short heptad repeats forming leucine zipper domains, and several others such as DNA polymerase, DNA topoisomerase, seryl tRNA synthetase, etc. (Lupas, 1996a). Long stretches of heptad repeats such as the rod domains of IF proteins are believed to form coiled coil structure, providing a hydrophobic seal on the helical surface and enabling the coiling between the two molecules. However, short heptad repeats are less promising to form coiled-coils (Lupas, 1996b). Recently, Kammerer et al. suggested that a distinct 13~14-residue "trigger" sequence is required to mediate proper assembly of the heptad repeats into a parallel homodimeric coiled-coil (Kammerer et al., 1998; Steinmetz et al., 1998). The four and a half heptad repeats of pinin do not seem to contain the "trigger" sequence. Therefore, it is doubtful that this heptad repeat domain in pinin can actually form coiled-coil *in vivo*.

A glycine loop domain was recognized adjacent to the heptad repeats region. Glycine loops are tandem quasi-repeating peptides that are rich in glycines. Each such peptide usually contains motifs with an aromatic residue followed by several consecutive glycines interspersed by occasionally hydrophilic residues such as serine, asparagines and arginines. Or sometimes, the motif is composed of an aromatic residue followed by only one or two glycines and /or a long-chain aliphatic residue. The patterns of glycine loop domains are highly variable in exact sequence and they intend to form highly flexible β-turns. Glycine loops have been found widespread in three classes of proteins, IF proteins, loricrin—major envelope components of terminally differentiated epithelial cells, and single-stranded RNA binding proteins (Steinert et al., 1991). Pinin seems to contain a glycine loop domain with three glycine loop motifs.

Furthermore, a glutamate-rich α-helical domain and a "QLQP" repeats domain compose the central part of pinin cDNA. Glutamate residues confer the potential α-helical domain highly rich of negative charges. However, motif homology of this region has not yet been characterized. The "QLQP" domain contains a long stretch of repeats with glutamine residue interspersed alternately with either leucine or proline, or in several occasions with serine or alanine. The frequent presence of proline residues resembles of the proline-rich motif (minimal consensus sequence: PXXP, other consensus motifs: RPLPXXP, XPXXPXK, etc.) of SH3 domain binding site (Alexandropoulos et al., 1995; Wang et al., 2000). Additionally, a group of small proteins rich in proline residues are also found in the cornified cell envelope of terminal differentiated epithelial cells cross-linking with other cornified cell envelope components via lysine and glutamine residue (Steinert et al., 1998; Steinert and Marekov, 1995). It is worth mentioning that desmoplakin and envoplakin, two desmosomal components, are also found in cornified cell (Steinert et al., 1998). However, it is uncertain what the precise function of this "QLQP" domain in pinin is.

At the carboxyl terminal end of pinin, there is a poly-serine domain followed by highly positively charged DRK repeats. In addition, several RS dipeptides/tetrapeptide, one of the features of splicing factor SR proteins, sparsely spread within both the poly-serine domain and the DRK repeats domain. Poly-serine domain has been found in several nuclear phosphoproteins (Blencowe et al., 1998; Zimowska et al., 1997) and it is believed to contain potential phosphorylation sites. Multiple kinase recognition motifs have been recognized in pinin and most of them are within the poly-serine domain (Kemp and Pearson, 1990). Data from two-dimensional gel analyses (unpublished) presented

distinctive spots before and after *in vitro* phosphatase treatment, suggesting that pinin is a phosphoprotein. Although there are RS containing motifs at the carboxyl terminus of pinin, pinin probably is not a typical SR protein since it does not contain recognizable RNA binding motif. However, the poly-serine stretch and the RS containing property may put pinin in line with other RS containing proteins such as SRm160 and SRm300 (Blencowe et al., 2000; Blencowe et al., 1998).

Sequence analysis also indicated the presence of several canonical import consensus motifs as well as leucine/hydrophobic residue-rich domains that could potentially facilitate nuclear export. Two canonical nuclear localization signals were also found at either the amino end or the carboxyl end. This may provide an explanation of the dual location of pinin. However, experimental evidence is needed to demonstrate the actual transport of pinin between the cytoplasm and the nucleus.

A possible role of pinin in the desmosome-IF complex organization. Pinin was initially identified as a desmosome-IF associated protein that was dynamically recruited to pre-formed desmosomes, but was absent from nascent desmosomes (Ouyang and Sugrue, 1992). Immunofluorescence analyses demonstrated a distribution of pinin at the lateral surface of numerous types of epithelial cells co-localizing with a constitutive desmosomal plaque component desmoplakin. In addition, pinin was also observed to co-localize with keratin filaments at the desmosome. Further immuno-EM studies confirmed the immunofluorescence observation that pinin was shown to reside at the cytoplasmic face of desmosomal plaque where intermediate filaments (IFs) converge upon desmosomes. Meanwhile, adhesion assembly assays presented an interesting correlation between pinin's assembly to the desmosome and the organization of IFs.

When MDCK cells were cultured under the low calcium condition for about 36 hours, both desmoplakin and pinin were seen diffusely distributed in the cytosol. However, after changing the cells back to normal calcium media, desmoplakin was seen to assemble to cell-cell boundary significantly before pinin. When keratin was examined at different time points together with pinin and desmoplakin, the organization of keratin filaments seemed to be enhanced at desmosomes after the recruitment of pinin to the desmosomes. These results indicated that pinin probably is not integral for desmosome assembly, however, it may play an important role in or at least is correlated to the organization/stabilization of desmosome-IF complex.

Nuclear localization of pinin and evidence for possible function of pinin in the nucleus. The localization of pinin in the nucleus was first noticed in transiently transfected culture cells as well as in several carcinoma derived cell lines (Shi and Sugrue, 1996). Others also demonstrated residence of pinin/DRS in the nucleus of cultured cells and in various tissues by immunofluorescence approach using antibodies against synthetic peptides representing the amino acid sequences deduced from *Xenopus Laevis* cDNA (Brandner et al., 1997). It was claimed that pinin/DRS was an exclusive nuclear protein. However, Pin Ouyang (Ouyang, 1999) reported contrary data presenting different scenario of pinin's locations. It was shown that there exists at least three isoforms in MDCK cells, pinin 1 desmosomal isoform (pinin 1d), pinin 1 nuclear isoform (pinin 1n), and pinin 2. Two location-specific monoclonal antibodies generated against bacterially expressed pinin fusion proteins individually recognize either desmosomal pinin isoform or nuclear pinin isoform. It was declared the pinin is a moonlighting protein playing roles varying with its subcellular locations and interacting partners. In

addition, polyclonal antibody 3A, generated against pinin GST-fusion protein also stained MDCK cells at the desmosome and in the nucleus (Ouyang and Sugrue, 1996). Recently, a polyclonal antibody UF 215 generated against the amino end domain of pinin (residues 1-165) was employed in an immunofluorescence study to examine endogenously expressed pinin in MDCK cells. Both cell-cell boundary staining and nuclear foci staining were seen in the same cell (Appendix I). Therefore, it is establishing that pinin is a protein with dual locations in the cell under the given circumstances.

Limited information has been reported on possible function of pinin in the nucleus. Nevertheless, Brandner et al (Brandner et al., 1998) reported several interesting observations. Double immunofluorescence exhibited the co-localization of pinin/DRS with SC35--a splicing factor, and with Sm-proteins—a general constituent of snRNPs, in the "speckled" domains of the nucleus, but not with Sm-proteins and collin present in coiled bodies. Furthermore, upon treatment of the cells with RNA-polymerase II inhibitor α-amanitin, pinin/DRS appeared to be located in the same category of nuclear subdomains positive for Sm-proteins. In addition, pinin/DRS was co-fractionated with splicing factor SF3a, SF3b, and 17S U2 snRNP in biochemical analysis of the nuclear extract, indicating that pinin may be one of the components of a multiunit protein complex involved in pre-mRNA splicing activities. Taken together, an involvement of pinin in the pre-mRNA splicing activities was implied, however, evidence pertaining to the molecular contact of pinin in the nucleus remains absent.

A potential tumor suppressive function of pinin. Although there is no commonly accepted definition for tumor suppressor genes, it is generally believed that tumor suppressors play essential roles as negative regulators in the multi-stage

development of cancers. In most of the cases, tumor suppressors would be down-regulated in developed tumors, although over-expression of putative tumor suppressor genes is not unusual as a compensatory mechanism to circumventing the disrupted regulatory pathways by overexpressing wild-type tumor suppressors. Additionally, one tumor suppressor may only present the loss-of function in specific subset of cancers. Nevertheless, reduced expression of certain tumor suppressor genes would ultimately result in situations favorable to the development of cancers, while consistent over-expression of tumor suppressors would be expected to reverse or prevent the tumor developing process in particular situations.

Accumulated evidence has suggested that pinin may function as a tumor suppressor. First of all, over-expression of pinin in cultured cells was correlated with the increase of cell-cell adhesion and inhibition of tumor-specific anchorage-independent growth. In specific, when HEK-293 cells were transit transfected with full-length cDNA of pinin, a striking phenotype alteration from cells with fibroblast-like spindle shape to intimately tightened cell islands was observed. Intriguingly, the cell-cell adhesion array seemed to be increased as represented by improvement of tight junctions and desmosomes (Ouyang and Sugrue, 1996). Transitional cell carcinoma derived cell line J82 was also transiently transfected with pinin cDNA. As a consequence, J82 cell lose the ability of anchorage-independent growth (Shi and Sugrue, 2000). When pinin cDNA antisense was transfected into MDCK cells, the typical intimately adherent epithelial cells became spindle-shaped resembling fibroblast cells. In addition, FISH (Fluorescence In Situ Hybridization) analysis indicated the location of pinin gene is at 14q13 and further alignments of STS markers more precisely located the gene within a previously identified

tumor suppressor locus D14S75-D14S228 (Chang et al., 1995; Shi and Sugrue, 2000). Furthermore, northern blot analyses revealed diminished mRNA level of pinin in renal cell carcinomas (RCC) as well as in other cell lines and immuno-histochemical examination of various patients' tumor samples reflected absence or greatly reduced pinin expression in transitional cell carcinoma (TCC) and in RCC (Shi and Sugrue, 2000). On the other hand, Degen *et al.* (Degen et al., 1999) reported the up-regulation of pinin/memA mRNA level in the progression of melanomas. Shi and Sugrue (Shi and Sugrue, 2000) observed increased level of pinin expression in a subset of RCC, suggesting the dysregulation of pinin may be related with a subset of cancers. Taken together, pinin was suggested to function as a tumor suppressor.

Intermediate Filaments and Related Proteins

Intermediate filaments (IFs), along with microfilaments and microtubules, represent the cytoskeletal filament systems that form the cytoskeleton of cells. IF proteins are not only found in the cytoplasm where they form intricate filament networks extending from the nuclear envelope towards the plasma membrane, but also in the nucleus as constituents of the nuclear lamina (Foisner and Wiche, 1991; Fuchs and Weber, 1994; Goldman et al., 1991; Steinert and Roop, 1988). Dramatic progress has been made in the understanding of the structural composition and dynamic assembly of IFs. However, the biological function of IFs remains largely unknown. Lines of data have suggested that IFs and IF associated proteins (IFAPs) constituents of deformable cellular latticeworks, imparting integrity and strength to tissues throughout the body. Additionally, the discovery of human diseases caused by mutations in IF protein keratins (Corden and

McLean, 1996; McLean and Lane, 1995) and in IFAP proteins such as plectin (Gache et al., 1996; McLean et al., 1996; Smith et al., 1996) reflect the important mechanical role of IFs as well as the roles played by IFAPs to link to the rest of cytoskeleton. Currently, IFs are believed to carry out their functions through their mechanical and dynamic properties regulated by complex and largely unknown mechanisms involving linkages to the cell surface, the nuclear envelope and other cytoskeletal elements (Chou et al., 1997; Goldman et al., 1999).

Intermediate filament protein superfamily. IF proteins constitute a large family of more than 50 gene products that share a common characteristic structure. The overall IF proteins only have 20-30% of sequence homology (Fuchs and Weber, 1994). However, the extent of sequence homology, the pattern of cell type specific expression, and the similarity of exon-intron gene structures classified IF proteins into six different types (Goldman et al., 1999).

The largest group of IF proteins are keratins that expressed mainly in epithelial cells. There are at least 30 keratins ranging in size from 40 to 67 KD (Moll et al., 1982). Type I keratins are acidic (pKi = 4-6) including eleven epithelial proteins, K9-K20, and four hair keratins, Ha1-Ha4. Type II keratins are basic (pKi = 6-8), including eight epithelial proteins, K1-K8, plus four hair keratins, Hb1-Hb4. Most epithelial cells express at least one type I and one type II keratin since they are obligatory copolymers for forming keratin filaments (Goldman et al., 1999; Moll et al., 1982). For example, basal keratinocytes express K5/K14 with little if any K1/K10, whereas suprabasal keratinocytes lose most if not all of their K5/K14 and express K1/K10. Simple epithelial

cells as found in the liver, exocrine pancreas, intestine and kidney express K8/K18 with various levels of K19 and K20 (Fuchs and Weber, 1994; Steinert and Roop, 1988).

Vimentin, desmin, glial fibrillary acidic protein (GFAP), and peripherin are four known type III IF proteins. Vimentin is most widely expressed in mesenchymal cell types and in a variety of transformed cell lines and in tumors. In addition, vimentin often forms a scaffold IF network before the expression and assembly of differentiation-specific IF proteins such as desmin, GFAP, and peripherin (Goldman et al., 1999). Desmin is more restricted in smooth muscle and in skeletal and cardiac muscle cells. GFAP is expressed in glial cells and peripherin is found in the peripheral nervous system.

Type IV IF proteins refer to the three kinds of neurofilament constituents,NF-L (light), NF-M (medium), NF-H (heavy) as well as α-internexin. Type V IF proteins including lamin A, B, and C compose the nuclear lamina. At last, nestin—a protein expressed in proliferating stem cell of the developing mammalian central nervous system and to a lesser extent (and only transiently) in developing skeletal muscle, and Filensin—a protein expressed during differentiation of the vertebrate lens epithelial cells are present members of type VI IF proteins.

Structural property of IF proteins and their involvement in IFs assembly/stabilization. The unifying secondary structural principle of the IF proteins family is the presence of a tripartite motif: a central ~310-residue long α-helical rod domain and flanking non-helical head and tail domains. The α-helical rod domain is subdivided into four segments helix 1A, 1B, 2A, and 2B by non-helical linkers, L1, L1-2 and L2 (Fuchs and Hanukoglu, 1983).

Although sequence identity among all IF proteins is relatively low, two highly conserved consensus motifs are found either at the start of helix 1A or near the end of helix 2B. Deletion analyses and *in vitro* peptide-interference-assembly-assays have demonstrated that these two consensus motifs are essential for IF polymerization (Albers and Fuchs, 1989; Coulombe et al., 1990; Hatzfeld and Weber, 1992). High occurrences of mutation are also found in both of these two consensuses in IF related genetic disease although the rest of molecule has also been found as the target of some mutations (Corden and McLean, 1996; McLean and Lane, 1995).

Throughout the central rod domain are heptad repeats, coiled-coil structure, which provides a hydrophobic seal on the helical surface, enabling the coiling between two IF polypeptides. Interesting but not yet fully understood, there are two "stutters" in the heptad phasing at the center of segment 2B resulted from the apparent deletion of three residues (Parry and Steinert, 1995). The "stutters" are highly conserved so that they were thought to have structural/functional significance. However, there is no requirement in the conformation modeling that demands a kink in the axis of the coiled-coil structure. Interestingly, stutters in the coiled-coil of hemagglutinin have been shown to produce an underwinding of the supercoil (Brown et al., 1996). The local unwinding caused by the specific break of heptad repeat may have global effect on the structure and can modify both the assembly of the protein as well as its interaction properties (Brown et al., 1996).

A notable charge periodicity with alternating acidic and basic residues appearing at ~9.5-residue intervals along the rod was speculated to form electrostatic interactions that stabilize association between coiled-coil dimers or higher-ordered structures (Conway and Parry, 1990). In some cases, many of the acidic and basic residues are spaced 4 aa

apart, and such spacing is optimal for formation of ionic salt bridges, which can stabilize intrachain α-helices (Huyghues-Despointes et al., 1993).

The non-helical head and tail segments of IF proteins vary in length and amino acid composition. Length variations are greatest in the tail, which ranges from 9 residues in K19 (Stasiak et al., 1989) to 1491 in nestin (Lendahl et al., 1990). The low homology among the head and tail domain indicates, most of the cases, no common role played by them in structural features shared among IFs (Rogers et al., 1995). However, published data also indicate that the head domain of some specific IF proteins, such as NF protein, seem to enhance both end-end and lateral associations of IFs assembly (Heins et al., 1993) and the tail domains of some type III or type IV IF proteins primarily may be involved in lateral interactions (Goldman et al., 1999; Ip et al., 1985a; Ip et al., 1985b; Shoeman et al., 1990). It is always speculated that the less conserved end domains may be involved in the cell type specific functions of IFs as well as their higher order structure (Goldman et al., 1999).

Intermediate filaments associated proteins (IFAPs). As introduced above, IFs are closely associated with the cell surface, the nuclear envelope, and other cytoskeletal elements such as microfilaments and microtubules. These associations are mediated by a growing list of IFAPs that play important roles in IFs organization as well as cytoskeleton stabilization (Foisner and Wiche, 1991; Fuchs and Weber, 1994; Goldman et al., 1999; Steinert and Roop, 1988). In most of the literature, IFAPs refers to all proteins meeting one or more of the following criteria: cellular codistribution with IFs; occurrence at IF anchorage sites; copurification with IFs in vitro; binding to IFs or subunit proteins; and effects on filament organization or assembly (Foisner and Wiche, 1991).

IFAPs can be approximately classified as following: (a) some low molecular weight IFAPs such as fillagrin, which bind IFs into tight arrays by simple ionic/or H-bonding interactions (Aynardi et al., 1984). (b) high molecular weight IFAPs such as synemin (Becker et al., 1995; Bellin et al., 1999), paranemin (Hemken et al., 1997), nestin (Lendahl et al., 1990), tanabin (Hemmati-Brivanlou et al., 1992), which organize the IFs into loose arrays. (c) adhesion junctional components connecting to IFs. Demosomal proteins such as desmoplakin (Troyanovsky et al., 1993), plectin (Foisner et al., 1988) and plakophilin (Hatzfeld et al., 1994) have been shown to be able to directly bind to IF proteins and possibly function as linkers between the desmosome and IFs. Periplakin /envoplakin (Ruhrberg et al., 1997; Ruhrberg et al., 1996) and pinin (Ouyang and Sugrue, 1992) may also belong to this subgroup. Similarly, hemidesmosomal protein bullous pemphigoid antigen 1 (BPAG 1) and BPAG 2 could also be classified to this category. (d) cytoskeletal linkers. For instance, plectin (Allen and Shah, 1999), BPAG 1n/3n (Yang et al., 1999; Yang et al., 1996), and fimbrin (Correia et al., 1999) possess both IF and actin binding sites, while plectin and BPAG 3n contain microtubule binding domain. It is worth mentioning that, desmoplakin, plectin, periplakin, envoplakin and BPAG 1/2 share similar structure as "dumbbell" that they are grouped as "plakin" family proteins (Kowalczyk et al., 1999). The significance of IFAPs in cytoskeletal integrity was highlighted by the correlation of muscular dystrophy associated with epidermolysis bullosa simplex (MD-EBS) with the expression of truncated plectin and by studies of BPAG 1-null mice and patients afflicted with MD-EBS (Gache et al., 1996; McLean et al., 1996; Smith et al., 1996).

IFs and cytoskeleton network. It has been generally accepted that the maintenance of IF network requires intact microfilaments and microtubules system. Previously, IFs was only considered to play a limited role, if any, in the organization of microtubules and microfilaments. However, data from *in vitro* peptide-interfering-assembly-assay showed that, when vimentin peptides were introduced into the cells, they induced rapid disruption of vimentin IF networks in fibroblasts, accompanied with the rounding-up of cells and the disassembly of both microtubules and microfilaments (Goldman et al., 1996). This result indicated the interdependence of the whole cytoskeleton system, especially underscored the role played by intermediate filaments. Moreover, plectin and BPAG 1n/BPAG 3n, both are IFAPs and are members of "plakin" gene family, have been demonstrated to function as a linker protein bridging between IFs and microfilaments and/or microtubules (Svitkina et al., 1996; Yang et al., 1999; Yang et al., 1996). It is possible that IF, together with IFAPs, play pivotal roles in linking the three cytoskeletal elements into interdependent functional units (Goldman et al., 1999).

Desmosomes

Desmosomes are major intercellular junctions locating at the lateral membrane of cells in epithelia, myocardium, and arachnoid. They are intimately involved in maintaining the structural and functional integrity of tissues by serving as adhesive complexes and as lateral membrane attachment sites for intermediate filaments (Kowalczyk et al., 1999). Biological significance of desmosomes is illustrated in certain epidermal blistering diseases where desmosomal glycoproteins are autoantibody targets (Hashimoto et al., 1995) and in inherited diseases correlated with mutations of

desmosomal proteins plakophilin (McGrath et al., 1997) and desmoplakin (Armstrong et al., 1999). Recently, emerging evidence also indicated that desmosomal components might play roles not only in cell adhesion, but also in the intracellular signal transduction (Kowalczyk et al., 1999).

Structural components of desmosomes and distinctive functions of desmosomal proteins. A desmosome, ultrastructurally, appears as a "spot weld" between adjacent cells with a central core region sandwiched by two symmetrical electron-dense cytoplasmic plaques (Kelly and Shienvold, 1976). Bundles of intermediate filaments extend toward the plasma membrane, loop through the plaques, and back towards the cytoplasm. The central core region consists of overlapping domains of transmembrane glycoproteins, desmogleins and desmocollins (Mathur et al., 1994). The cytoplasmic plaques composition is more complicated, and can be classified as two categories: constitutive proteins, such as desmoplakin, plakoglobin, plakophilin, and non-constitutive components that are also named as desmosome-associated proteins, including plectin (Koszka et al., 1985), envoplakin, periplakin, and pinin. The constitutive components of desmosomes are integral for the assembly and stabilization of desmosomes and cell-cell adhesions as demonstrated by the studies of null-mice lacking either desmoplakin (Gallicano et al., 1998) or plakoglobin (Bierkamp et al., 1996). The desmosome-associated proteins more likely play roles in stabilizing or strengthening the connections between desmosomes and the intermediate filaments (Kowalczyk et al., 1999). It is worth pointing out that, plakophilin 1/2/3 have been reported to localize in the nucleus as well as at the desmosome (Bonne et al., 1999; Mertens et al., 1996), which is very similar to the subcellular distribution of pinin. Lacking of expression of

plakophilin 1 was correlated with poorly differentiating cells such as tumors (Moll et al., 1997). Intriguingly, plakoglobin and plakophilins are members of the armadillo gene family, and the arm repeats motif has been shown to be the site mediating protein-protein interactions in signaling pathways (Huber et al., 1997).

Protein linkage within the desmosome-IF complex. Biochemical analysis and transfection studies on desmosomal proteins have revealed complicated multiple modes of protein-protein interactions in the desmosome-IF complex. These dynamically regulated interactions play substantial roles in the assembly and organization/stabilization of the complex as well as in cell-cell adhesions.

Desmoglein and desmocollin are two transmembrane desmosomal cadherins. *In vitro* binding assays demonstrated the direct interactions of either or both of desmoglein and desmocollin with desmoplakin (Fuchs and Cleveland, 1998; Meng et al., 1997), plakophilin (Fuchs and Cleveland, 1998), or plakoglobin (Chitaev et al., 1996; Fuchs and Cleveland, 1998; Mathur et al., 1994; Wiche et al., 1993) in an isoform specific manner. On the other hand, desmoplakin has been shown to interact with plakoglobin via its amino terminal head and bind to IFs by its carboxyl terminal domain (Fuchs and Cleveland, 1998; Troyanovsky et al., 1996; Wahl et al., 1996; Witcher et al., 1996). Plakophilin 1 was also shown to directly interact with IFs in an overlay binding assay (Fuchs and Cleveland, 1998; Hatzfeld et al., 1994), although most recently, immuno-EM studies revealed plakophilin 1 localizing quite close to the plasma membrane, rather than in the region of intermediate filaments anchoring (Alison North, 1999). In addition, plectin (Foisner et al., 1988), envoplakin, periplakin (Ruhrberg et al., 1997; Ruhrberg et al., 1996) and pinin (Ouyang and Sugrue, 1992), which have all been

localized to the desmosome periphery, have been suggested to directly or indirectly interact with IFs.

Desmosome integrity vs. intermediate filaments stabilization. It has been argued whether structural integrity of desmosmoes affects the assembly/stabilization of IFs, or *vice versa*. Transfection analyses showed that the transfected carboxyl terminal domain of desmoplakin, which is deficient of the plakoglobin binding domain, co-localized and ultimately resulted in the complete disruption of IFs of the cell (Green et al., 1992). Similar, disruption of IFs was also observed in cells transfected with truncated desmocollin lacking of the plakoglobin-binding-domain. Desmoplakin null embryo, which proceeded through implantation but did not survive beyond E6.5 stage of the development, caused disorganization of IFs and dramatic reduction in the number of desmosome-like junctions (Gallicano et al., 1998). These data suggested that the defect of desmosomal components may dramatically influence the stabilization of IFs. On the other hand, several studies have provided evidence showing that desmosome plaque components are assembled in the cytoplasm attaching to or in close association with keratin IF (Green et al., 1987; Jones and Goldman, 1985; Pasdar and Nelson, 1988a; Pasdar and Nelson, 1988b), indicating a requirement of IFs for the formation of desmosomes. However, in keratin 18 null mice, hepatocyte desmosomes have a typical appearance and size distribution of desmosomes in the absent of IFs, suggesting that at least in the liver of K18 null mice, no IF was required for the formation of desmosomes (Gallicano et al., 1998).

Nuclear Matrix and Nuclear Subdomains

Mammalian cell nucleus is a three-dimensional mosaic complex composed of condensed chromatin, interchromatin regions, nucleolar compartments, and a surrounding double-membraned nuclear envelope containing nuclear pore complexes. Nuclear matrix, depicted as a dynamic fibro-granular structure surrounded by nuclear lamina, is believed to confer the shape of the nucleus as well as influence the nuclear functions by organizing the nuclear chromatin and scaffolding the structural organizations of many important intra-nuclear events such as DNA replication, transcription, post-transcription RNA processing (Berezney et al., 1995; Hughes and Cohen, 1999; Nickerson et al., 1995). Molecular studies and high-resolution morphological approaches allow for the observations of numerous nuclear subdomains and the sites of the occurrence of the genetic nuclear activities. It is noticed that the individual structural domains are associated with specific genetic functional loci and the associations between these various domains and loci are dynamic and can change in response to specific cellular signals (Matera, 1998; Matera, 1999). Therefore, current view on the relationship between the structure and the function of the nucleus is: internal nuclear framework may actively enhance gene expression by integrating and regulating assembly and cascade of nuclear events, DNA replication, RNA transcription and RNA processing machinery components could diffusionally arrive to the sites of gene readout with some aspects of nuclear structures in responding to gene expression (Pederson, 1998).

Ultrastructure of the nuclear matrix. After the specific biochemical preparations (Jackson and Cook, 1988; Nickerson et al., 1997; Wan et al., 1999), nuclear matrix generally is left as two parts: the nuclear lamina, which is known as a protein shell

primarily constructed of lamin A, B, and C (Georgatos et al., 1994; Gerace et al., 1984), and the internal nuclear matrix, morphologically presented as a network of core filaments intimately connecting to and suspending particles and granular elements while bounded by the meshwork of the nuclear lamina (Fey et al., 1986). The largest particles are the nucleoli, while the rest may correspond to various nuclear functional subdomains, including DNA replication foci, transcription foci, coiled bodies, gems, speckled domains, RNA transcript track and domains, and PML bodies (Lamond and Earnshaw, 1999; Matera, 1999; Nickerson et al., 1995). Under the EM examination, the space between the chromatin contains two types of ribonucleoprotein-containing elements: perichromatin fibrils and interchromatin granule clusters, which have subsequently been functionally related to the sites of pre-mRNA transcription and processing (Spector, 1993).

The core filaments. The observations of filaments in the nucleus during studies on the nuclear matrix preparation have been reported over the years. Berezney and Coffey (Berezney and Coffey, 1974) had noticed filaments that were abundant and heterogeneous in diameter in RNP-depleted nuclear matrix. Comings and Okada (Comings and Okada, 1976) studied on the mouse liver nuclear matrix preparations and revealed two classes of filaments with diameters of 2-3 nm and 10-30 nm. Jackson and Cook (Jackson and Cook, 1988) and Hozak et al (Hozak et al., 1995) had revealed a three-dimensional network of core filaments that were 10-11 nm in diameter. Recently, Nickerson et al (Nickerson et al., 1997; Wan et al., 1999) published a newly modified nuclear matrix preparation protocol uncovered that the internal matrix structural fibers were built on an underlying network of branched 10 nm core filaments. Efforts have

been made to identify the protein subunit of the core filaments. lamin A, a relative of IF protein family, has been reported to stain some nuclear foci in Hela cells as well as in erythroleukemia cells (Hozak et al., 1995; Neri et al., 1999). However, no filament-like staining has been seen using antibodies against any known protein. The protein composition of the core filaments remains to be determined.

Speckled domains and coiled bodies. Functional domains in the nucleus appear as dense bodies enmeshing in the extensive network of matrix core filaments in resinless section of nuclear matrix preparations (Nickerson and Penman, 1992). These domains are stained as multiple "foci" (in some literature called "speckles") at the level of immunofluorescence. They are very dynamic and can be distingushed by their unique protein or RNA components.

Generally, mammalian nuclei contain 20-50 speckled domains. Nuclear speckles were first detected by the staining patterns of autoimmune patient sera that recognize protein or RNA components of snRNPs (Perraud et al., 1979; Spector, 1993). In addition to snRNPs, speckled domains are also highly enriched in non-snRNP splicing factors SR proteins (Spector, 1993). However, nascent transcripts, detected by [^{3}H] Br-UTP, do not seem to co-localize with speckled domains (interchromatin granules) but rather coincide with the perichromatin fibril region. When assuming pre-mRNA splicing takes place cotranscriptionally, this data indicated that the splicing activity might occur adjacent but outside of the "speckles". This view is supported by the observation of splicing factors "movement" in between interchromatin granules and perichromatin fibrils upon the initiation or inhibition of transcription (Carmo-Fonseca et al., 1992). Currently, speckled

domains are considered as the sites for storage of splicing factor rather than the sites for active pre-mRNA splicing.

There are approximately 1-5 coiled bodies per cell nucleus, which can be identified by a constitutive marker protein p80 coilin (Andrade et al., 1991; Raska et al., 1991). At present, coiled bodies, which is also named Cajal bodies (Gall et al., 1999), are known to contain three major classes of snRNPs, including spliceosomal U1, U2, U3, U4, U5, and U6 snRNPs (Carmo-Fonseca et al., 1991; Huang and Spector, 1992; Matera and Ward, 1993), U7 snRNP required for 3' end processing of histone mRNA (Woo et al., 1996; Wu et al., 1993), and U3/U8 small nuceolar RNAs (snoRNAs) involving in processing of pre-rRNA (Bauer et al., 1994; Wu et al., 1993). Additionally, coiled bodies have been located near the replication histone gene clusters (Frey and Matera, 1995) and were shown to preferentially associated with snoRNA genes (Schul et al., 1999). Nascent snRNPs do not accumulate in coiled bodies while matured or maturing snRNPs are highly concentrated in them (Schul et al., 1998). Furthermore, sequential targeting of snRNPs from coiled bodies to speckled domains was reported in several recent studies (Gall et al., 1999; Sleeman and Lamond, 1999). Taken together, it was suggested that coiled bodies might be involved in the biogenesis/maturation of snRNP (Matera, 1999). Interestingly, besides the RNA processing related components, transcription factors and cell-cycle factors have also been found in coiled bodies (Grande et al., 1997; Jordan et al., 1997), indicating coiled bodies may have functions other than involving in snRNP biogenesis.

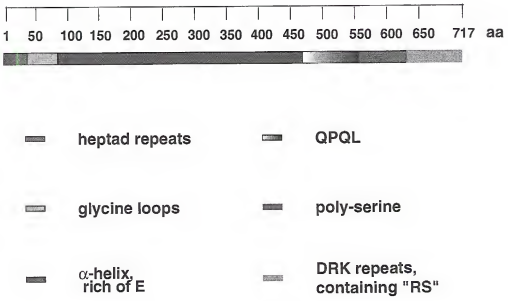

Figure 1.1 Diagram of pinin domains predicted from conceptual translation product of cDNA.

CHAPTER 2
TWO-HYBRID SCREENING IDENTIFIED POTENTIAL PROTEIN PARTNERS OF PININ AT THE ADHESION/CYTOSKELETAL ANCHORAGE COMPLEX AS WELL AS IN THE NUCLEUS

Introduction

Pinin is a phosphoprotein identified by a monoclonal antibody O8L generated against the insoluble fraction of MDCK cells (Ouyang and Sugrue, 1992). Preliminary Immunofluorescence studies located pinin at the cell-cell boundary coinciding with desmosomal protein desmoplakin in cultured cells as well as in various tissues. Further immuno-EM studies illustrated pinin's presence at the sites adjacent to where intermediate filaments converge to desmosomes. Consistent with this view, double-immunofluorescence found the colocalization of pinin with keratin at the cell-cell boundary while keratin filaments network was as usual extending from nuclear envelope to the plasma membrane (Ouyang and Sugrue, 1992; Ouyang and Sugrue, 1996). Therefore, pinin appears to be a desmosome-IF-associated protein.

The subcellular distribution of pinin appears to be dynamic under certain circumstances. Pinin was recruited to pre-formed desmosomes but was absent at nascent desmosomes (Ouyang and Sugrue, 1992). When MDCK cells and cornea epithelial cells were wounded, pinin staining at the desmosomes was greatly reduced and pinin was seen diffusely distributing in the cytosol. During the process of wound healing, pinin seemed

to return back to the desmosomes in parallel with decreased cell-cell migration (Shi and Sugue, 2000b).

Phenotype alterations resulted from the expression of sense or antisense pinin cDNA in cultured cells indicated an involvement of pinin in cell-cell adhesion and in the establishment/maintenance of cell polarity. HEK 293 cells, when transfected with cDNA of pinin, exhibited a phenotype change from spindle shape to more intensely associated cells as islands (Ouyang and Sugrue, 1996). However, when antisense of pinin cDNA was transfected into MDCK cells, the typical epithelial cell polarity was lost that the transfected cells exhibited a spindle shape phenotype characteristic of fibroblast cells. It is important for us to understand the molecular emchanisms involved in these modifications.

Pinin is also observed to be present in the nucleus of cultured cell lines and in various tissues (Brandner et al., 1997; Brandner et al., 1998). In fact, pinin is believed to have dual locations in the cell at the desmosome-IF as well as in the nucleus (Ouyang, 1999) In addition, biochemical fractionation analysis indicated the co-fractionation of pinin with specific splicing complexes. Double immunofluorescence illustrated a co-localization of pinin with Sm protein and SC35 in nuclear "speckled" domains. Possibly, pinin is involved in nuclear splicing related activities (Brandner et al., 1998). It will be significant and interesting to elucidate molecular mechanisms for pinin's translocation into the nucleus and for the role pinin plays in the nucleus.

With the known information and the corresponding concerns about pinin, it was thought that, to identify the proteins interacting with pinin would provide significant indications on the possible protein linkage pinin may be involved in and render the

opportunities to reveal pinin functions and molecular mechanisms involved by pinin. Based on the multifunctional nature of pinin, it was thought that pinin might be involved in multiple protein-protein associations. Accordingly, yeast two-hybrid screenings were performed to identify protein partners of pinin.

Yeast two-hybrid system has been widely and successfully used as a method to detect protein-protein interactions (Chien et al., 1991; Fields and Song, 1989). It relies on the modular nature of many site-specific transcription activators, which consist of a DNA-binding domain and a transcription activation domain. The DNA-binding domain serves to target the activator to the specific gene to be expressed, while the activation domain contacts other proteins of the transcription machinery to enable transcription to occur. A two-hybrid system is based on the observation that the two domains of the activator need not to be covalently linked but can be brought together by the interaction of any two proteins. Therefore, the application of this system requires two hybrids to be made: (1) A DNA-binding domain fused with one protein (bait). (2) A transcription activation domain fused to either another protein or to a cDNA library (prey).

Yeast two-hybrid system has been widely and successfully used elsewhere. The relatively high sensitivity of this system and library-scale screening allow for a better detection to the possible protein-protein interactions than most other methods. Furthermore, the cDNA clone for any interacting protein identified is immediately available from the library. In some cases, the clone identified may only encode part of the protein that the domain responsible for the interaction could be apparent from the initial screening (Phizicky and Fields, 1995). Conveniently, the proteins are synthesized by yeast from the cDNA clones. No biochemical purification is required, although some

proteins may not be able to experience normal post-translational modification and/or correctly fold in the yeast. However, it was believed that protein complex assembly of adhesion junctions is positively regulated by dephosphorylation of junctional components. Presumably, the lack of phosphorylation in the yeast would not inauspiciously interferes with our effort to identify pinin partners. Another problem of the two-hybrid system is the relatively high occurrence of false-positives. The interactions are made to occur by artificially bringing every protein into nucleus. Some proteins that never have any chance to meet each other in their real lives might be brought together. Another reason for the occurrence of false-positives is the leaking of the reporter genes. As a matter of fact, the later shortcoming is very obvious in the previously available yeast two-hybrid systems. Recently, a modified yeast strain PJ69-4A (James et al., 1996) has been reported to be able to improve the system dramatically by bringing in a third more stringent reporter gene, Adenine 2, in addition to Histidine 3 and LacZ (Fig. 2.1). In the following described two-hybrid screenings, yeast strain PJ69-4A was employed instead of yeast strain Y190 or CG1945 provided by Clontech two-hybrid Matchmaker II system.

Materials and Methods

Yeast strain and media. The *Saccharomyces Cerevisiae* strain PJ69-4A (*MATa trp1-901 leu2-3, 112 ura3-52 his3-200 gal4 gal80D LYS2::GAL1-HIS3 GAL2-ADE2 met::GAL7-lacZ*) (James et al., 1996) was used in all the two-hybrid assays. The yeast was grown on synthetic media (SD) with appropriate omission of amino acid for plasmid selection. Tryptophan and leucine were selective markers for the co-transformed bait and

prey plasmids. Histidine 3, Adenine 2 and lac Z are reporter genes for interaction occurrence between GAL4-BD and GAL4-AD. In "-HIS" medium, histidine was omitted as well as tryptophan and leucine, while in "-Ade" medium, adenine was omitted as well as typtophan and leucine. In addition, 1 mM 3-amino-triazole (1 mM 3-AT) was added in all the media to inhibit the auto-activation of histidine3 reporter gene.

Plasmid constructions. Yeast shuttle vector plasmid pAS2-1 (Clontech, Matchmaker II) contains Gal4 DNA-binding domain and tryptophan selectable marker was used in the baits construction. Human pinin cDNA amino terminal portion (residues 1-480) and carboxyl-terminal portion (residues 470-717) were amplified by PCR (NEB, Vent polymerase) with primers STS124 (GCA CAT ATG ATG GCG GTC GCC GTG AGA ACT) and STS123 (GCG CGT CGA CTG AGC CTG AGG TTG AGC CAC), STS125(GCA CAT ATG GAA TCT GAG CCC CAA CCT GAG) and STS122 (CGC CGT CGA CAT TAA CGC CTT TTG TCT TTC CTG T). The PCR product was then subcloned into the Nde I/Sal I site of pAS2-1, fused with the GAL4-DNA binding domain and used as the bait in the yeast two-hybrid screenings. Sequencing of the bait constructs was conducted to ensure the fidelity of the clones.

Quick and simple yeast transformation. A simplified protocol was employed to transform plasmid(s) into the yeast host. Yeast PJ69-4A freshly growing on a plate were collected and washed with sddH_2O once. The yeast were suspended in 0.1 M LiAc, incubated for 5 min in 30°C, then collected as pellet. Following reagents were added into the tube in the order of 240 µl 50% PEG (MW 3400), 36 µl 1 M LiAc, 25 µl 2 µg/µl single strand DNA, 5 µl of plasmid containing 100-1000ng DNA, and 45 µl sddH_2O. Thereafter, the tubes were vigorously vortexed and incubated in 30°C for 30 min, then

heat shocked for 20 min. Finally, the yeast were collected by centrifugation and re-suspended in sterile TE buffer, spread on appropriate solid media. (http://www.umanitoba.ca/academic/faculties/medicine/biochem/gietz/Quick.html.).

Protein extract from the yeast. Glass beads lysis method was applied in this study to extract the whole cell proteins from yeast. Specifically, 2 ODs unit (volume x OD600) of yeast overnight culture were collected, mixed with 1/10 volume of cold 100% TCA, and placed on ice for 5 min. The yeast were harvested by centrifugation at 25,000 rpm, 10 min, then washed once with cold 10% TCA. The pellets were suspended in 100 µl 10% TCA, and then transferred to tubes containing 0.25 g acid washed 0.5 mm glass beads, vortex for 1min, on ice for 1 min, and vortex again for 1 min. Thereafter, the yeast lysate were transferred to new tubes while they were already precipitated by 10% TCA. The TCA precipitate was collected by spinning at top speed for 5 min at 4^0C. The pellets were washed with 1.0% TCA, solublized in 100 µl SDS-PAGE sample buffer, boiled for 5 min, and ready for SDS-PAGE analysis.

Western blot. Western blot was employed to monitor the expression of bait in the yeast (Fig. 2.2 and Fig. 2.3). Protein extract of the yeast PJ69-4A transformed with any of the bait constructs was subjected to SDS-PAGE and transferred to a nitrocellulose membrane for routine western blot. The blots were incubated with anti-GAL4 DNA binding domain monoclonal antibody following the manufacturer's instruction (Clontech), and the recognition of antigen by antibody was visualized by ECL (Amersham). Positive control was the yeast PJ69-4A transformed with plasmid pAS2-1, which contains built-in GAL4 DNA binding domain. Negative control was the plain yeast PJ69-4A.

Determination of the 3-AT concentration optimal for the reporter gene selections. Transformants containing the bait construct were streaked on a series of SD/-W, -H plates containing 3-AT with the concentration of 0, 0.5, 1, 1.5, 2, 2.5, and 3 mM. The lowest concentration that didn't allow any growth of the yeast was 1 mM, which was selected as the concentration for the rest of the two-hybrid screening.

Tests for false-activation. Plasmid pGAD 10, which was used to construct the Matchmaker cDNA library, was transformed into yeast PJ69-4A that has contained the bait plasmid. The yeast transformants were plated on SD/-W, -L, +H, +a, + 1mM 3-AT plates and grew for 3-4 days. Then the transformants were streaked on the SD/-W, -L, -H, +a, + 1mM 3-AT plate. After one week, no colony was seen on the plates, indicating that the bait together with the library vector pGAD 10 did not cause any activation of the reporter genes. The yeast containing only the bait plasmid was also streaked on a SD/ -W, +L, -H, +a, +1mM 3-AT plate. No growth of any colony was observed on the plate, suggesting the bait itself was not capable of activating the reporter genes, either.

Amplification of a Matchmaker cDNA library. Clontech Matchmaker human fetal kidney cDNA library was titered and plated on 25 LB/amp plates at high density that the resulting colonies reached nearly confluent (~40,000 cfu per 150 mm plate). The plates were incubated at 37°C for 18-20 hr. 5 ml of LB/glycerol was added to each of the plates and the colonies were scraped up into the liquid, pooled in one flask, mix thoroughly. One-third of the mixture was subjected to CsCl gradient plasmid preparation (Stephen P. Sugrue lab protocol). The acquired plasmid DNAs are used for the library-scale transformations.

Library-scale yeast transformation. Yeast containing bait plasmid grew overnight in a volume appropriate for the scale of transformation. On the second day, the yeast cell titer was determined and inoculated into YPD media (Clontech) at the concentration of 5x 10^6 cells/ml. Yeast are left for growing at 30°C for 4-6 hr until the cell titer reached 2 x 10^7 cells/ml. The cells were harvested by centrifugation at 3,000g for 5 min, washed in 1/2 volume of sddH_2O and kept as cell pellet. The following components were added on top of the yeast cell pellet in the order of 240 µl 50% PEG (MW 3400), 36 µl 1 M LiAc, 25 µl 2 µg/µl single strand DNA, 1 µl of library plasmid containing 1 µg DNA, and 49 µl sddH_2O, per reaction for one 150 mm plate. 1 µg of the library plasmid DNA resulted in a transformation efficiency of 2-4 x 10^4 yeast colonies on one 150 mm plate, which was the highest efficiency achieved by testing a series of concentrations of library plasmid DNA. The mixture of all transformation components were vigorously suspended and incubated at 30°C for 30 min, and then heat shocked at 42°C for 20 min by inverting the tubes for 15 sec after every 5 min. At last, the yeast cells were collected by centrifugation and resuspended in 1 ml of sdd H_2O (sterile distilled and de-ionic water) per reaction, spread on one 150 mm plate (Gietz et al., 1997). (http://www.umanitoba.ca/academic/faculties/medicine/biochem/gietz/2HS.html)

Two-hybrid screening. Approximately 10^6 independent yeast colonies were screened by sequentially transforming pinin N' bait (1-480)/pinin C' bait (470-717) and the library plasmid DNA into the yeast host PJ69-4A. The transformants were first put onto -HIS media (SD/-W, -L, -H, +a, +1mM 3-AT) selecting for 14 days. Then the yeast colonies growing on the plates were replicated onto –Ade media (SD/-W, -L, +H, -a, +1mM 3-AT), selecting for 5 days. Thereafter, liquid culture ONPG β-galactosidase

assay (Clontech Matchmaker II user's manual) was applied onto the positive colonies survived from both -HIS and -Ade selections. β-galactosidase assay positive clone plasmids were retrieved from the yeast and subjected to extensive controls. The final positive clones were sequenced and applied to BLAST search analyses (Table 2.1).

Retrieval of shuttle plasmid from yeast. 2 ml of overnight culture grown from one colony on a selective plate were collected and washed in 1 ml sdd H_2O. The cell pellet was resuspended in 100 ml of TENS buffer (100 mM NaCl, 10 mM Tris·HCl, pH 8, 1 mM EDTA, 0.1% SDS) and transferred to tubes with 0.25 g acid washed glass beads, vortexed for 1 min. 100 µl of phenol was added in the tube and the tube was vortexed again for 1 min, spin at top speed for 2 min. 150 µl of the aqueous phase was transferred to a new tube and phenol extracted for one more time. 100 µl of the aqueous phase was transferred and incubated together with suspension beads (QIAGEN, Gel Extraction Kit) for 5 min, washed the beads with PE washing buffer (QIAGEN, Gel Extraction Kit), and finally the plasmid DNA was recovered from the beads into 20 µl ddH_2O.

Controls for the two-hybrid screening identified clones. The plasmids of the clones positive for -HIS, -Ade and β-gal assay selections in the two-hybrid screens were tested for false-positive by controls (Table 2.2). Individual of the prey clone plasmids was co-transformed into yeast host with each of the following plasmids containing various GAL4 fusion protein as alternative bait: pVA3-1 (GAL4-p53), pLAM5'-1 (GAL4-lamin C), GAL4-pinin (1-480), GAL4-pinin (470-717). In addition, the prey clones were also individually transformed into the yeast. All these transformants were selected on -HIS media. Any growth on the selective media may indicate the existence of a false-positive.

Results

Two-hybrid screenings identified two groups of proteins interacting with pinin. Approximately 10^6 independent clones were screened with either N' pinin (1-480) or C' pinin (470-717). The transformants were subjected to sequential triple selections, -His, -Ade, and β-gal assays (Table 2.1). Evidently, -Ade selection was the most stringent selection in that it greatly reduced the false-positives that had escaped from the selection of HIS 3 reporter gene and the positive clones from the -Ade selection was >90% positive in the β-gal assays. Through sequence analyses and extensive controls, 21 clones were identified to interact with pinin N' bait and 22 clones were identified to interact with pinin C' bait. Intriguingly, most of the 21 N' bait interacting clones fall in to a category of adhesion junction/cytoskeleton anchorage complex protein, and the characterized proteins among the 22 C' bait interacting clones are pre-mRNA splicing related (Table 2.3 and 2.4). This result is consistent with our previous morphological observations that pinin has dual locations at the desmosome-IF and in the nucleus.

K18, K8 and K19 directly interacted with the N-terminal Domain of pinin in the two-hybrid screen. Among the 21 N' bait interacting clones, five were cytokeratin 18 (K18), one was cytokeratin 8 (K8), and one was cytokeratin 19 (K19). K18, K8, and K19 are three cytokeratins that form keratin intermediate filaments in the simple epithelial cells (Steinert and Roop, 1988). This, consistent with the morphological observation of pinin colocalizing with keratin at the desmosome, suggested that pinin was capable of directly binding to keratin filaments protein subunit. More data pertaining to keratin-pinin interaction will be discussed in chapter three.

A periplakin-like protein and a trichohyalin-like protein were identified to interact with the N-terminal domain of pinin in the two-hybrid screen. Two clones were identified that encoded one gene containing motifs homologous to periplakin. We named the gene periplakin-like protein. Periplakin is a desmosome-associated protein existing in the cornified cell envelope of differentiated epithelial cells (Ruhrberg et al., 1997) and it is a member of the plakin family that includes desmoplakin, envoplakin, BPAG1/2, and plectin (Kowalczyk et al., 1999). Another clone identified has homology to trichohyalin, which is an intermediate filaments-associated protein that associates in regular arrays with keratin filaments in the granular layer of the epidermis as well as in inner root sheath cells of hair follicles (Lee et al., 1993). Trichohyalin is also cross-linked in the cornified cell envelope of differentiated epidermis (Steinert, 1995, 1998). Although the EST database provided more sequence segments either upstream or downstream of the coding region of the clone, the full-length cDNAs of both of the genes have not yet been characterized. Nevertheless, it is tempting to surmise that these two uncharacterized proteins are desmosome-IF associated proteins and pinin may bind to them under the certain circumstances.

Both exo 70 isoform and syntaxin 4 are capable of binding to pinin N' domain in the two-hyybrid screen. Two clones containing different but overlapping regionsa of an Exo 70 isoform and one clone containing part of syntaxin 4 were identified by pinin N' bait in the two-hybrid screen. Exo 70 is one of components in exocyst complex (Sec6/8p complex) (Hsu et al., 1998; Kee et al., 1997). Exocyst complex has been suggested to specify the delivery of vesicles containing lateral membrane proteins to the sites of cell-cell contact and induce vesicle-membrane fusion at

specific domain of the membrane (Grindstaff et al., 1998; Hazuka et al., 1999; Hsu et al., 1999). Syntaxin 4 is one member of the t-SNARE family defined by a conserved 60-amino acid "t-SNARE" homology domain. Syntaxin 4 is dominantly expressed on the basolateral membrane domain of MDCK cells, hepatocytes, and pancreatic acinar cells (Fujita et al., 1998; Low et al., 1998), and it is involved in binding to v-SNARE for the docking and fusion of secretory vesicles (Calakos et al., 1994, Linial, 1997). Both exocyst and syntaxin 4 was once co-precipitated by an antibody against one component of exocyst complex (Hsu et al., 1996; Ting et al., 1995), and both of them are integral in the biogenesis of epithelial cell polarity (Hsu et al., 1999; Yeaman et al., 1999). If pinin indeed binds to these two proteins *in vivo*, it may provide an interpretation for previous observed phenotype alteration in the pinin sense and antisense transfection assays.

AKAP 350 was identified to bind to pinin N-terminal domain in the two-hybrid screen. A 500bp clone containing protein kinase A RII (regulatory subunit II) binding site of AKAP350 (A-Kinase-Anchoring-Protein 350) (Schmidt et al., 1999) was isolated twice from the library by the N' bait. AKAPs are a family of proteins that contain a structurally conserved RII binding domain through which AKAP sequestrates protein kinase A (PKA) to the particular subcellular locations (Colledge and Scott, 1999; Pawson and Scott, 1997; Scott and McCartney, 1994). AKAPs has been found in almost every subcellular compartment including mitochondria, peroxisomes, Golgi apparatus, endoplasmic reticulum, centrosomes, nucleus, cell membrane periphery as well as microtubules. A specific targeting domain of each AKAP decides the location of the particular AKAP. It is believed that an AKAP serves as a scaffold protein for the second messenger response related signaling by placing PKA holoenzyme at locations of

activities and by scaffolding signaling molecules into one protein complex (Klauck et al., 1996; Colledge, 1999). AKAP350 has been localized to centrosomes as well as to the cell-cell boundary (Schmidt et al., 1999). Immunofluorescence has shown the co-localization of pinin with AKAP350 at the cell boundary in various tissues and at the boundary as well as at the centrosomes in liver cells (unpublished data, also see Appendix II). Pinin molecule contains multiple PKA recognition motifs. It would be interesting to investigate if and how pinin involves in PKA regulatory activities in pinin function related events.

I-mf was identified to bind to pinin N' bait in the two-hybrid screen. Pinin N' bait also identified a clone encoding protein I-mf, which is a myogenic repressor that associates with MyoD family members so as to retain them in the cytoplasm by masking their nuclear localization signals (Chen et al., 1996). As introduced in chapter one, pinin is a protein with dual locations at the desmosome-IF and in the nucleus. Pinin contains at least two canonical nuclear localization signals as well as potential export signals. A question waiting for an answer is how the translocation of pinin is regulated. If I-mf could bind to pinin *in vivo*, a similar mechanism for MyoD family proteins could be applied to pinin. This possibility is currently under investigation.

SRp75, SRm300 and a hypothetical SR protein were identified to bind to pinin N' terminal domain in the two-hybrid screen. One clone matched to SRp75, two clones matched to SRm300, and one clone matched to a hypothetical SR protein have been identified by the C-terminal domain of pinin. SRp75 is a SR protein that has been characterized to be involved in pre-mRNA splicing (Tacke and Manley, 1999; Zahler et al., 1993b). SRm300 is a subunit of a splicing coactivator that by itself does not

interfere with the splicing activity, but intimately associates with SRm160—the subunit of the splicing coactivatior that can stimulate pre-mRNA splicing activity (Blencowe et al., 2000; Blencowe et al., 1998; Eldridge et al., 1999). The hypothetical SR protein is an uncharacterized protein referred as hypothetical protein in the database. The majority of the database available sequence was included in the identified clone, which contains high content of RS dipeptide/tetrapeptides. Chapter four presents more data and discussion on the interactions of pinin with these three proteins.

Several motifs were found to be able to bind to pinin C-terminal domain. Some clones that were identified to interact with C-terminal domain of pinin in the two-hybrid screen contained only short ORF in frame with the GAL4 DNA binding domain. No significant homology among them has been found from the database analyses. However, sequence analyses revealed that they might be grouped in to "poly-proline" containing proteins, K (lysine), R (arginine), E (glutamate), G (glycine), repeats containing proteins, and phenylalanine (F) rich proteins (Table 2.5). It is tempting to surmise that these repeat sequences represent the protein motifs that could potentially interact with pinin C-terminal domain.

Discussion

In this study, two-hybrid screenings identified two groups of proteins that were capable of binding to pinin. Proteins interacting with the N-terminal domain of pinin include IF protein keratins, two potential desmosomal-IF complex proteins, cell polarity related proteins exo70 (isoform) and syntaxin 4, PKA signaling scaffold protein AKAP 350, and NLSs masking protein I-mf. Proteins interacting with the C-terminal domain of

pinin included SRp75, SRm300, and a hypothetical SRK protein. In addition, several groups of short ORFs were identified, which may be motif candidates that could interact with pinin C-terminal domain. These results are incredibly consistent with the dual location observation of pinin, and additionally present the possible protein-protein interactions pinin may be involved in.

Most of the proteins in the cells play their roles in a protein complex by interacting with other component(s) at a given moment. The identification of the partner(s) that possibly interact with the studied protein could be very helpful for further investigations on the function(s) of the protein. Results from this study suggest: (1) pinin might play roles in desmosome-IF association by binding to keratins and potential desmosomal proteins. (2) pinin may be involved in regulation of cell polarity formation via its interaction with exo 70 isoform and syntaxin 4. (3) pinin may directly bind to SRp75 and SRm300, and play a role in pre-mRNA splicing related events. (4) pinin's activities may be intersected with PKA signaling pathway or PKA regulatory mechanism. (5) a potential nuclear transport regulatory mechanism involved by I-mf was suggested.

The biological significance of protein-protein interactions detected in two-hybrid system has always been a serious concern since the interaction of the two proteins examined in the nucleus of the yeast is not necessarily meaningful *in vivo*. Generally, one or more independent assays will be employed to confirm the biochemical interaction and the physiological possibility in cells. Various *in vitro* binding assays, such as solid phase binding assay, overlay binding assay, etc. have been widely utilized to test for the direct biochemical interaction possibility. Co-immunofluorescence and co-immunoprecipitation are always employed to demonstrate the possible coincidence of the

two proteins in cells. We understand that we can not draw any conclusion about the biological significance of those interactions with pinin until both *in vitro* and in cell assays provide confirming evidence. This rule is going to be applied to all subsequent studies.

In the next two chapters, I will present the studies focusing on two groups of pinin interacting proteins, keratins at the desmosome-IF complex and RS-containing proteins in the nucleus. Additionally, tempting hypotheses and discussions are proposed in chapter five.

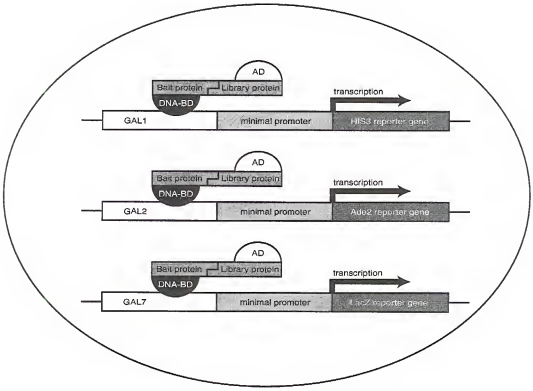

Figure 2.1 The reporter gene structure in the yeast strain PJ69-4A.

MAVAVRTLQEQLEKAKESLKNVDENIRKLTGRDPNDVRPIQARLLALSGPGGGR

GRGSLLLRRGFSDSGGGPPAKQRDLEGAVSRLGGERRTRRESRQESDPEDDDVK

KPALQSSVVATSKERTRRDLIQDQNMDEKGKQRNRRIFGLLMGTLQKFKQESTV

ATERQKRRQEIEQKLEVQAEEERKQVENERRELFEERRAKQTELRLLEQKVELA

QLQEEWNEHNAKIIKYIRTKTKPHLFYIPGRMCPATQKLIEESQRKMNALFEGR

RIEFAEQINKMEARPRRQSMKEKEHQVVRNEEQKAEQEEGKVAQREEELEETGN

QHNDVEIEEAGEEEEKEIAIVHSDAEKEQEEEEQKQEMEVKMEEETEVRESEKQ

QDSQPEEVMDVLEMVENVKHVIADQEVMETNRVESVEPSENEASKELEPEMEFE

IEPDKECKSLSPGKENVSALDMEKESEEKEEKESEPQPEPVAQPQPQS

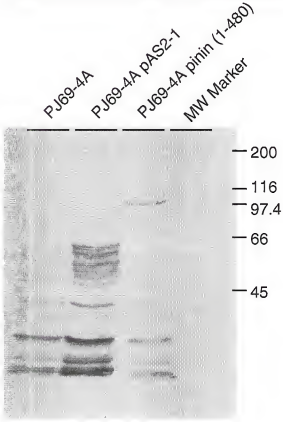

Figure 2.2 Western blot demonstrated the expression of pinin N' bait (residues 1-480, as shown above) in the yeast strain PJ69-4A. Monoclonal antibody against the GAL4-DNA-binding-domain was used to detect the whole cell lysate of the three different yeast: PJ69-4A, PJ69-4A transformed with empty vector pAS2-1, and PJ69-4A transformed with pinin (1-480). A band with the size of ~100 KD is seen in the lane of PJ69-4A pinin (1-480), which represents the expressed pinin N' bait.

PQPEPVAQPQPQSQPQLQLQSQSQPVLQSQPPSQPEDLSLAVLQPTPQVTQ

EQGHLLPERKDFPVESVKLTEVPVEPVLTVHPESKSKTKTRSRSRGRARNK

TSKSRSRSSSSSSSSSSTSSSSGSSSSSGSSSSRSSSSSSSSTSGSSGRDS

SSSTSSSSESRSRSRGRHNRDRKHRRSVDRKRRDTSGLERSHKSSKGGSSR

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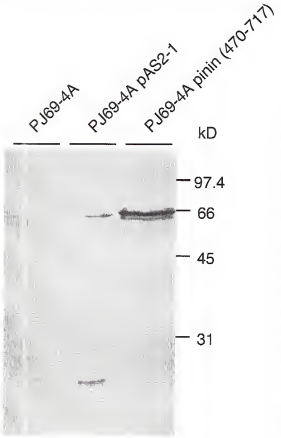


Figure 2.3 Western blot demonstrated the expression of pinin C' bait (residues 470-717, as shown above) in the yeast strain PJ69-4A. Monoclonal antibody agaist GAL4-DNA-binding-domain was used to detect the whole cell lysate from the three different yeast: non-transformed, transformed with empty vector pAS2-1, and transformed with pinin (470-717). A band with the size of 66 KD was seen in the lane of PJ69-4A pinin (470-717), which represents the expressed pinin C' bait.

	N' Bait Pinin (1-480)	C' Bait Pinin (470-717)
Independent Library Clones Screened	$> 10^6$	$> 10^6$
Positive clones from the -HIS Selection	10^5	10^5
Positive Clones from the -Ade Selection	57	119
Positive Clones from ONPG β-gal assay	51	98
Non-redundant clones sequenced	27	34
Clones containing ORF	21	22

Table 2.1 Flow chart of the two-hybrid screenings presents the selection progress of the clones. More than 10^6 independent clones from the matchmaker human fetal kidney cDNA library were screened with either the N' pinin (1-480) or the C' pinin (470-717) as bait. The transformants were subjected to -His selction, -Ade selection, and β-gal assay, sequentially. Final positives were sequenced.

Yeast	PJ-69-4A					
Bait	N' bait	C' bait	pAS2-1	pVA3-1	pLAM5'-1	none
positive clones						
N-1-1	++	-	-	-	-	-
N-3-1	++	-	-	-	+	-
N-6-2	++	-	-	-	-	-
N-15-1	++	-	-	-	+	-
N-16-1	++	-	-	-	+	-
N32-3	++	-	-	-	-	-
N-35-1	++	-	-	-	-	-
N-36-1	++	-	-	-	-	-
N-37-2	++	-	-	-	-	-
N-55-4	++	-	-	-	+	-
N-59-1	++	-	-	-	+	-
N-65-3	++	-	-	-	+	-
N-70-1	++	-	-	-	-	-
N-72-1	++	-	-	-	+	-
N-73-2	++	-	-	-	-	-
N-75-1	++	-	-	-	-	-
C-15-8	-	++	-	-	-	-
C-25-10	-	++	-	-	-	-
C-29-1	-	++	-	-	-	+
C-34-3	-	++	-	-	-	-
C-54-1	-	++	-	-	-	-

Table 2.2 Various controls were employed to test the possible false-positive clones identified from the two-hybrid screens. Prey plasmids were individually cotransformed with one of the following bait: N' bait (pinin residues 1-480), C' bait (pinin residues 470-717), empty vector pAS2-1, pVA3-1 (GAL4-p53), pLAM5'-1 (GAL4-lamin C), or without any bait (none). Growth of the transformants on -HIS and -Ade media were reflected by the "+" and "-". A few clones cotransforming with GAL4-lamin C resulted in the growth of the yeast. Thoes clones turned out to be keratins (See Table 2.3). Clone C-29-1 resulted in the growth of the yeast by itself, therefore, it was a false-positive.

Clone	Identity
N-3-1	Keratin-18 from 1 to 430 of 430aa
N-15-1	Keratin-18 from 1 to 430 of 430aa
N-16-1	Keratin-18 from 1 to 430 of 430aa
N-55-4	Keratin-18 from 1 to 430 of 430aa
N-59-1	Keratin-18 from 1 to 430 of 430aa
N-65-3	Keratin-8 from 120 to 387 of 483aa
N-72-1	Keratin-19 from 69 to 400 of 400aa
N-37-2	Periplakin-like
N-70-1	Periplakin-like
N-36-1	Trichohyalin-like
N-35-1	Exo 70 isoform from 1 to 152aa
N-75-1	Exo 70 isoform from 1 to >152aa
N-32-3	Syntaxin 4 from 200 to 297 of 297aa
N-1-1	AKAP350 from 2106 to 2271 of 3524 aa
N-73-2	AKAP350 from 2106 to 2271 of 3524 aa
N-6-2	I-mf from 41 to 200 of 246 aa

Table 2.3 Identification of N' pinin domain (1-480) binding partners by a two-hybrid screening. 21 interacting clones were isolated, sequenced, and identified by BLAST database alignment. 16 of the clones encoded either complete or partial sequence of proteins listed in the BLAST database.

Clone	Identity
C-54-1	SRp75 from 117 to 494 of 494aa
C-15-8	SRK hypothetical protein from 17 to299 of >299aa
C-25-10	RNA binding protein from 129 to 712 of 2752aa
C-34-3	RNA binding protein from 1 to > 200 of 2752aa

Table 2.4 Identification of C' terminal pinin domain (470-717) binding partners by a two-hybrid screening. 22 interacting clones were isolated, sequenced, and identified by BLAST database alignment. Four clones coded for domains of proteins listed in the BLAST database.

Clones	Characteristics
C-18-1	P repeats
C-23-3	P repeats
C-25-3	P repeats
C-43-3	P repeats
C-59-1	P repeats
C-73-1	P repeats
C-74-1	P repeats
C-23-1	P repeats
C-25-13	P repeats
C-71-1	P repeats
C-16-2	K, R, E, G, repeats
C-17-3	K, R, E, G, repeats
C-9-1	K, R, E, G, repeats
C-37-4	K, R, E, G, repeats
C-4-2	K, R, E, G, repeats
C-28-1	F rich
C-7-1	F rich
C-33-1	N.D.

Table 2.5 Clones identified by C-terminal domain of pinin (residues 470-717) contain uncharacterized repeat sequence in their coding region. Among the 22 pinin 470-717 interacting clones, 10 clones are almost identical coding sequence that contains proline repeats, 5 clones are different coding regions but contain K, R, E, G, repeats, 2 clones are phenylalanine rich in the sequence. These repeats sequence could be potential pinin-binding motifs.

CHAPTER 3

DISSECTION OF PROTEIN LINKAGE BETWEEN KERATIN AND PININ, A PROTEIN WITH DUAL LOCATION AT DESMOSOME-INTERMEDIATE FILAMENTS COMPLEX AND IN THE NUCLEUS

Introduction

Pinin was first identified to be a desmosome-associated protein, which was recruited to the preformed desmosomes of the epithelia, but was absent at nascent desmosomes (Ouyang and Sugrue, 1992). Immunofluorescence and immuno-EM studies have shown pinin decorating keratin filaments near the cytoplasmic face of the desmosomal plaque in the vicinity of keratin filament convergence upon the desmosome. Our previous studies have correlated the placement of pinin at the desmosome with increase in the organization/stabilization of desmosome-IF complex (Ouyang and Sugrue, 1992; Ouyang and Sugrue, 1996). Presumably, one of the functions of pinin is related to the desmosome-IFs complex.

The expression level of pinin has been correlated with the overall epithelial phenotype. HEK-293 cells, when transfected with pinin full-length cDNA, exhibited a striking phenotype change from a fibroblast-like spindle shape to cells with extensive cell-cell contact growing in culture as islands (Ouyang and Sugrue, 1996). Intriguingly, EM analysis of these transfected cells revealed that the array of epithelial cell junctions was enhanced as demonstrated by the increase of both desmosomes and tight junctions. In addition, carcinoma derived cells, when transfected with pinin cDNA, exhibited

inhibition of anchorage-independent growth in soft agar. Furthermore, pinin's gene locus and dysregulation in primary tumor tissues suggest that pinin may function as a tumor suppressor in certain types of cancer (Degen et al., 1999; Shi and Sugrue, 2000a).

Pinin has also been localized in the nucleus in various tissues as well as in cultured cell lines [(Brandner et al., 1997; Brandner et al., 1998; Ouyang, 1999). A possible involvement of pinin in spliceosome function was proposed by Brandner, et al. (Brandner et al., 1998). The dual location of pinin may be indicative of the involvement of pinin in multiple cellular activities both at the desmosome and in the nucleus, however, it is not yet clear whether or not the function of pinin in cell-cell adhesion is coordinated with its function in the nucleus. As a step toward understanding the functions of pinin, we sought to identify proteins that interact with pinin. In this study, we focus on the ability of pinin to bind keratin.

Keratin filaments are anchored to the lateral plasma membrane at desmosomes. These intercellular junctions reinforce epithelial adhesion as well as integrate the IF network across the entire epithelium. Numerous structure-function studies of desmosomal proteins have revealed details pertaining to the molecular organization of desmosome-IFs complex. The relationships among the desmosomal components have been extensively reviewed elsewhere (Fuchs et al., 1997; Kowalczyk et al., 1999; Smith and Fuchs, 1998; Troyanovsky and Leube, 1998). The constitutive components of the desmosome include desmosomal cadherins (desmogleins and desmocollins) and plaque proteins, plakoglobin, desmoplakin, and plakophilin. Among these proteins, desmoplakin (Kouklis et al., 1994; Meng et al., 1997) and plakophilin (Hatzfeld et al., 1994; Smith and Fuchs, 1998) have been shown to bind directly to keratins. In addition, other peripherally

desmosome associated proteins such as plectin (Foisner et al., 1988; Wiche et al., 1993), envoplakin/periplakin (Ruhrberg et al., 1997; Ruhrberg et al., 1996) and pinin (Ouyang and Sugrue, 1992), are also thought to interact, directly or indirectly, with keratin. Significant questions pertaining to the molecular associations and specific roles of these accessory proteins of the desmosome are as of yet unresolved.

To identify potential protein-protein interactions of pinin, a two-hybrid screening was performed with either the amino portion or the carboxyl portion of pinin as bait. In this study, we presented a detail analysis on the binding of the amino end domain of pinin to one group of the identified proteins, the keratins. Keratin 18 (K18), keratin 8 (K8), and keratin 19 (K19) were shown to interact with the amino portion of pinin from the two-hybrid screen. Further truncation analyses defined the specific domain of keratin that mediates the interactions. In addition, the specific domain of pinin molecule sufficient for the interaction was characterized, and through site-directed mutagenesis, the essential residues within this particular domain were investigated. *In vitro* blot overlay assays were performed to confirm the interaction between the amino end domain of pinin and the keratins. Overall, our data strongly suggest that pinin is capable of binding directly to the intermediate filament proteins such as keratins. These data provide important information on eventual understanding of mechanism by which pinin may affect the assembly/stabilization of epithelial cell adhesion.

Materials and Methods

Yeast strain and media. See chapter two.

Bait construct and two-hybrid screening. The DNA fragment encoding for pinin residues 1-480 was obtained by PCR and cloned in-frame into the GAL4 DNA

binding domain (GAL4BD; bait) vector pAS2-1 (Clontech, Matchmaker II sysytem). The GAL4BD-pinin vector was cotransformed with a Clontech Matchmaker cDNA library into the yeast strain PJ69-4A using the yeast transformation method of Gietz et al. (Gietz et al., 1997). The library consisted of human fetal kidney cDNA fused to the activation domain of GAL4 (GAL4AD, prey) in the pGAD 10 vector (Clontech).

Approximately 10^6 transformants were screened, and first subjected to -HIS media. Then the yeast colonies growing on -HIS media were replicated to and selected on -Ade media. Positive colonies from -Ade selection were subjected to liquid culture ONPG β-galactosidase assay according to manufacturer's procedure (Clontech). A well-characterized interaction between p53 and SV40 large T-antigen was used as a positive control in β-gal assays. Baseline level of β-gal activity was determined from negative control yeast that had been cotransformed with GAL4-BD-pinin (residues 1-480) and GAL4-AD. Each value of β-gal units was decided by an average of enzyme activity of 3 independent positive colonies. The "prey" plasmids were recovered from triple positive (HIS, Ade, and LacZ) clones and co-transformed with the control heterologous bait, p53, pinin C' (residues 470-717) and GAL4-binding domain. In addition, the "prey" plasmid was also transformed by itself into the yeast host to test for possible false-positive. Putative positive clones that were selected from -HIS, -Ade and β-gal selection assays and exhibited no interaction with control bait were further subjected to sequence analysis.

To examine the ability of truncations of pinin to interact with keratins, the GAL4BD vector containing the individual pinin truncations or point mutation constructs were co-transformed with the pGAD10 vector containing keratin 18 into PJ69-4A yeast cells. To examine the ability of truncations of keratin 18 to interact with the amino end

of pinin, the original bait was co-transformed with individual truncations of keratin 18, fused to activation domain of GAL4 in the pGAD10, into PJ69-4A yeast cells. Triple selections described above were applied to all transformants.

Generation of pinin/keratin truncations and pinin point mutations. Truncations of pinin and truncations of keratin 18 were generated by PCR using the primer sets listed in Table I and Table II. PCR products of human pinin were fused in frame to the GAL4BD in the vector pAS2-1 at Nde I/Sal I sites. PCR products of human keratin 18 were fused in frame to the GAL4AD in the vector pGAD10 at Xho I/ EcoR I sites. Point mutations of the pinin amino end 1-480, fused in frame to GAL4BD in the pAS2-1 vector, were generated using the Quick Change Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA) with the primer sets listed in Table III.

Expression of pinin fusion protein in E. Coli. and generation of the polyclonal antibody against the pinin GST-fusion protein. Pinin residues 1-165 were obtained by PCR with primers STS 65 (5' CCG AAT TCC CGC TTC AGA GAG AAG ATG 3') and STS 61(5' CGC TCG AGG GCC TTT CAG TAG CAA CAG 3'). This PCR fragment was cloned in frame to vector pGEX-4T-3 (Pharmacia) at Xho I/EcoR I sites. The glutathione-S-transferase (GST) fusion protein GST-cp(1-165) was expressed in *Eschericha coli.* strain BL21 (Novagen) and purified with glutathione Sepharose 4B (Pharmacia) according to the manufacturer's instruction. Similarly, a mutant GST-fusion protein of the residues 1-165, GST-cp (1-165) L8P, with a substitution of leucine 8 by a proline , was generated with the Quick Change Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA), expressed and purified as described above.

The pinin DNA encoding for 5' end residue 1-165 was also cloned into pET 28(+) b (Novagen, pET system) and expressed as a T7 tagged and His_6-fusion protein in *Eschericha coli.* strain BL21 (Novagen). The fusion protein was affinity purified using the charged HIS·Bind metal chelation resin (Ni^{2+} beads) following the instructions of the manufacturer (Novagen, pET System Manual).

A polyclonal antibody, UF215, was generated using GST-cp(1-165) as an antigen by Cocalico Biologicals. Inc.. The specific immunoactivity of UF 215 to pinin amino domain was verified by western blot on pET System expressed His_6-fusion protein described above (data not shown).

Purification of keratin filament protein from MDCK cells. MDCK cells were grown to confluency in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum (Life Technologies, Inc.), 100 units/ml each of penicillin and streptomycin. Keratin proteins were then prepared from these cells according to a procedure described elsewhere (Aynardi et al., 1984; Zackroff et al., 1984) with slight modifications. Specifically, cells were rinsed rapidly in ice-cold PBS and then lysed in PBS containing 1 % Triton X-100, 0.6 M KCl, 1 mM $MgCl_2$, 5 mM EDTA, 5 mM EGTA, and the protease inhibitors: 1 mM phenymethylsulfonyl fluoride, 1 mM DTT, 1 mg/ml leupeptin, 1 mg/ml pepstatin A, 1 mg/ml aprotinin (Sigma). The extract was treated with DNAse (0.5 μg /ml) at 37^0C for 20 mins, and then was centrifuged at 2,000 g at 4 ^{0}C for 10 mins to pellet the IF-enriched cytoskeleton. To eliminate microfilament and microtubule components, the IF-enriched cytoskeleton extract was first washed with PBS in the addition of 5 mM EDTA, 0.5 mM PMSF, and 1 mM DTT, then washed with low salt buffer (60 mM KCl, 1 mM EDTA, 1 mM cysteine, 10 mM

ATP, 40 mM imidazole, pH 7.1), high salt buffer (0.6 M KCL, 1 mM EDTA, 2 mM ATP, 1 mM cysteine, 40 mM imidazole), and low salt buffer again. This KCl extracted pellet was dissolved in 8 M urea in 10 mM Tris·HCl buffer supplied with proteinase inhibitors and subjected to ultracentrifugation @ 125, 000 g for 1 hour, 4^0C. The supernatant was dialysed into 10 mM Tris·HCl, and frozen in -80^0C.

In vitro blot overlay binding assays. In vitro overlay binding assays were performed as described elsewhere with slight modifications (Smith and Fuchs, 1998). 2 μg of each of the purified keratins, bovine serum albumin (BSA), pinin amino end fragment 1-165, and mutant pinin 1-165 L8P were separated on a 10 % SDS-PAGE. The proteins were then transferred onto a nitrocellulose membrane, and blocked by incubation in reaction buffer (10 mM Tris·HCl, 150 mMNaCl, 1 mM $MgCl_2$, pH 7.4) with the addition of 0.1% (v/v) Tween 20 and 5% (w/v) non-fat milk powder at 4^0C overnight. The blot was washed the second day with the reaction buffer. Thereafter, the blot was incubated 4 hrs at room temperature with the bacterially expressed pinin amino end domain either wild type GST-cp-(1-165) or mutant GST-cp-(1-165) L8P. The concentration of the overlay proteins was 3 μg/ml in the reaction buffer with the addition of protease inhibitor cocktail (Boehringer Mannheim) and 0.1% Tween 20, 1% BSA, and 0.5% Triton x-100. After the overlay, the blots were washed thoroughly with several fresh changes of the reaction buffer, and subjected to routine western blotting using ECL method with a slight modification. Specifically, UF215 diluted 1:1000 in TBST (10 mM Tris·HCl, 150 mM NaCl, 1 mM $MgCl_2$, 0.1% Tween 20, pH 7.4) was used as primary antibody. 5% normal goat serum was applied for 30 mins before the secondary anti-rabbit antibody (Amersham) (1:10,000) incubation. Standard washes were applied in

between each step. Lastly, protein interactions on the blots were visualized by ECL method (Amersham). As a control for the overlay assay, GST was used instead of wild type pinin fusion protein, and subsequently probed with anti-GST antibody (Pharmacia) in a western blot.

Results

K18, K8, and K19 were identified in a yeast two-hybrid screening by the amino portion fragment of pinin. In an effort to identify proteins that bind to pinin amino terminal domain, a yeast two-hybrid screening on a human fetal kidney cDNA library (Clontech) using pinin (residues 1-480) as bait was performed. Of the approximately 10^6 transformants screened, 21 independent cDNA clones were isolated. The recovered prey plasmids were verified by co-transforming one of the plasmids with either GAL4-BD-pinin N' (residues 1-480) or control heterologous baits including GAL4-BD-p53, GAL-BD-pinin C' (residues 470-717), and GAL-BD. All the negative controls displayed no growth on the selective media, indicative that the prey plasmid interacting with the specific pinin bait resulted in the activation of the reporter genes. Characterization of the identified clones revealed that the most prevalent protein, which exhibited binding to the amino end of pinin, was keratin. Five of the identified clones encoded full-length of keratin 18 (residues 1-430), one encoded the rod domain of keratin 8 (residues 90-387), and another encoded the rod and the tail domain of keratin 19 (residues 69-400) (Fig. 3.1).

The 2B domain of keratin contains the binding site for pinin. K18, K8 and K19 are three keratins expressed in the simple epithelial cells. These keratins share common structural properties. Each possesses an amino end non-helical head domain, a

central coiled-coil α-helical domain, and a non-helical tail domain in various lengths (Fuchs and Weber, 1994; Steinert and Roop, 1988). Because pinin (residues 1-480) bound equally well to each of these keratin clones and the common domain shared by all was the rod domain, we surmised that the rod domain might contain the sufficient sequence for the interaction with pinin. To further map the binding site within keratin, truncation constructs coding either the coil 1 or the coil 2 of K18/K8/K19 were generated and examined for their ability to bind pinin in two-hybrid assays (Fig. 3.1). While the coil 1 constructs K18 (residues 69-240), K19 (residues 81-235), and K8 (residues 91-235) exhibited no significant binding to pinin (residues 1-480), coil 2 containing constructs K18 (residues 234-391), K19 (residues 244-390), and K8 (residues 260-381) all exhibited interaction with pinin. It was, however, noticed that the coil 2-pinin interactions were approximately 10-fold weaker than the interaction of the full-length rod domain as indicated by the β-gal assay. While reporter gene activity, such as β-gal, does not correspond linearly with the strength of interaction, these assays can be useful in estimating relative strength of interactions between similar molecules or domains. The data suggest that either some sequence outside coil 2 domain may contribute to the interaction or the longer constructs may present the binding domain of keratin in a more advantageous conformation for pinin-binding.

The carboxyl terminus of the 2B domain within the coil 2 contains a highly conserved consensus motif, suggested to be significant for assembly/stabilization of the intermediate filaments in cells (Albers and Fuchs, 1987; Albers and Fuchs, 1989; Hatzfeld et al., 1994; Hatzfeld and Weber, 1992; Kouklis et al., 1992). K18 (residues 69-276), which excluded the entire 2B domain, failed to interact with pinin (residues 1-480).

However, K18 (residues 69-372), which contained the majority of the rod domain but excluded the consensus motif, retained the ability to bind to pinin (residues 1-480) (Fig. 3.2). Taken this together with the results from Fig. 3.1, we concluded that the 2B domain of keratin contained the binding site for pinin.

Pinin residues 1-98 are sufficient for interacting with keratins. The amino end of pinin (residues 1-480) contains a short heptad repeat domain, a few glycine loops (Steinert et al., 1991), and a rather extensive glutamate rich α-helix domain (Ouyang and Sugrue, 1996). To more precisely map the domain of pinin that is sufficient for the interaction with keratins, five pinin truncation constructs were generated for two-hybrid analyses (Fig. 3.3). Constructs lacking the amino terminus of pinin (residues 85-480, 250-480 and 85-252) exhibited no significant interaction with keratin, while constructs (residues 1-252 and residues 1-98), which contained amino heptad repeats and glycine loops, exhibited binding to keratin.

Leucine 8 and leucine 19 within pinin are essential for binding to keratins. To further define the specific region of pinin domain that is essential for binding to keratins, site-directed mutageneses was employed. Leucine residues at position 8, 19 and 29, which were predicted to locate at either the "a" or "d" position of the heptad repeats within pinin (Berger et al., 1995; Lupas, 1996b; Lupas, 1997), were substituted with proline (N' L8P, N' L19P, and N' L29P). Interestingly, both N' L8P and N' L19P resulted in no growth at all on -Ade media (Fig. 3.4, A) and a baseline level of β-gal activity (Fig. 3.4, B), indicating the interaction between pinin and K18 was abolished with a single mutation. On the contrary, N' L29P retained the ability to grow on -Ade media, but the β-gal activity was somewhat reduced. One glycine within the predicted

first glycine loop of pinin was replaced by glutamate (N'G53Q). This subsitution, similarly to N'L29P, did not affect the viability of the transformed yeast, but a weaker interaction might have occurred as indicated by β-gal activity. Charged residues have been speculated to stabilize coiled-coil conformations. However, single substitutions of arginine 6 and lysine 28, with aspartate and glutamate, respectively (N' R6D, N' K28E) resulted in mildly dampened interactions (Fig 3.4). Therefore, leucine 8 and 19 were essential for pinin-keratin interaction. Leucine 29, glycine 53, arginine 6 and lyscine 28 were not as critical as leucine 8 and leucine 19, however, their involvement in the interaction could not be ruled out. Whether or not multiple (additive) substitutions of the residues would result in a more obvious affect on the pinin-keratin interaction is currently under investigation.

In vitro overlay binding assays verified the direct interaction between pinin amino end domain and keratins. Purified keratin from MDCK cells and bacterially-expressed pinin fragments, both wild type GST-cp-(1-165) and mutant GST-cp-(1-165) L8P, were utilized in the blot overlay binding analyses. Blots containing keratin preparations were overlayed with either wild type pinin GST-fusion protein GST-cp-(1-165), or mutant pinin GST-fusion protein GST-cp(1-165) L8P and subsequently reacted with UF 215 (Fig. 3.5 B). Only the wild type pinin construct exhibited binding to keratin, as visualized by its immunoreactivity with anti-pinin antisera UF215. The fact that the mutation L8P, which eliminated pinin-keratin binding in the two-hybrid assay, showed no binding in the overlay assay, provided strong support for the specificity of the *in vitro* binding assay and confirmed the observations from the two-hybrid assays. We concluded that the amino end domain of pinin was capable of directly binding to keratins.

Discussion

In this study, we present data demonstrating the direct interaction of the amino end domain of pinin with the 2B domain of keratins from simple epithelial cells. These data are not only consistent with our previous morphological observations, but provide biochemical support of pinin-IFs association.

There are four distinct coiled-coil stretches, 1A, 1B and 2A, 2B, in the central rod domain of a keratin molecule. Our data indicate that pinin binds to the sequence within the 2B domain of keratin. Coil 1 of keratin exhibited no binding to pinin, strongly supporting the conclusion that the interaction between the 2B domain of keratin and pinin amino terminal domain is indeed specific and not due to non-specific interaction of pinin with general coiled-coil containing proteins. Direct binding to the rod domain of keratins has been reported for BPAG 2, a hemidesmosome-associated protein, that binds to the 2B domain of K18 (Aho and Uitto, 1999). While desmoplakin has been shown to bind to the head domain of epidermal keratins such as K1/K10 and K5/K14 (Smith and Fuchs, 1998), it has also been noticed to be capable of binding to the rod domain of simple epithelial keratin K8/K18 heterodimer (Meng et al., 1997). Interestingly, point mutations within the 2B domain of keratins have been correlated with a subset human skin blistering diseases epidermolysis bullosa simplex although it is the 2B consensus motif that exhibits high occurrence of mutations (Chen et al., 1995; Chen et al., 1993; Hachisuka et al., 1995; McLean and Lane, 1995). This correlation to blistering phenotypes of 2B domain point-mutations including several cases occurring within the non-consensus region of the 2B domain may be indicative that, this domain of keratins and, in turn, the putative interactions of this domain with IF associated proteins such as

pinin, are important for the stabilization of the IFs-desmosome/IFs-hemidesmosome complex.

The truncation analyses suggested the amino end of pinin (residues 1-98) contained the sequence responsible for pinin binding to keratins. Although short coiled-coil of four to five heptad repeats have been reported (Lupas, 1997), it is not determined whether or not the four and half heptad repeats at the amino end of pinin are actually sufficient to form coiled-coil structure *in vivo*. Actually, the deficiency of "trigger" sequence (Kammerer et al., 1998; Steinmetz et al., 1998), which was determined to be necessary for the stable assembly of coiled-coil homodimers, is unfavorable for a coiled-coil type interaction between pinin and keratins. Data derived from point mutations of the amino terminal domain of pinin suggest the heptad repeats are essential for the interaction with keratins. However, while N'L8P and N' L19P completely abolished the binding of pinin to K18, N'L29P retained the ability to interact with K18 albeit a somewhat weaker interaction. The leucine 8 and 19 are more critical for the pinin-keratin interactions.

We have suggested that pinin may function as a tumor suppressor based on chromosomal location of pinin and tumor biological analyses (Shi and Sugrue, 2000a). It has been shown that the expression of pinin was absent or greatly reduced in certain carcinomas including renal cell carcinoma (RCC) and transitional cell carcinoma (TCC). On the other hand, pinin expression was seen up-regulated in melanoma cells (Degen et al., 1999) and a subset of RCC (Shi and Sugrue, 2000a). In vitro decreased pinin expression was correlated with loss of epithelial cell-cell adhesion, while increasing pinin expression was correlated with enhancement of cell-cell adhesion (Shi and Sugrue,

2000a). Interestingly, K18 and K8 have long been considered as cytological markers for carcinomas due to their persistent expression in tumor cells derived from simple epithelia and their aberrant expression in malignant progression of non-epithelial cells (Hendrix et al., 1992; Moll et al., 1982; Schussler et al., 1992; Trask et al., 1990). In addition, several studies suggested that K18/K8 filaments have a role in the tumorigenicity. For example, in K8 deficient mice, adult animals developed pronounced colorectal hyperplasia (Baribault et al., 1994) and the expression of K8 and K18 in human melanoma cell lines resulted in increased invasive and metastatic properties of the cells (Hendrix et al., 1992; Zarbo et al., 1990). It is tempting to speculate that the tumor suppressor function of pinin is related to the interaction of pinin with keratins.

This study did not address the issue regarding the relationship between the desmosome and pinin. Our initial two-hybrid screens identified many other, as of yet uncharacterized, proteins interacting with pinin N' bait 1-480 (unpublished data). These clones included one containing motifs that are highly homologous to periplakin (Ruhrberg et al., 1997), a desmosome-IFs associated protein forming cornified envelop in the stratified epithelial cells. The possibility of pinin connecting to desmosome through this periplakin-like protein is currently being addressed.

In summary, we have demonstrated that pinin can bind to keratin 18, keratin 8 and keratin 19 via its amino end domain. Specifically, the 2B domain of keratin contains the sequence mediating the interaction with pinin, and the amino end (residues 1-98) of pinin was sufficient to bind keratin and leucine 8 and 19 are essential for the interactions. Identification of the keratin as well as other protein binding domains of pinin will be integral steps for future studies. We believe that investigation on the function(s) of pinin

in cell adhesion and IF organization will greatly contribute to our current knowledge of epithelial cell-cell adhesion.

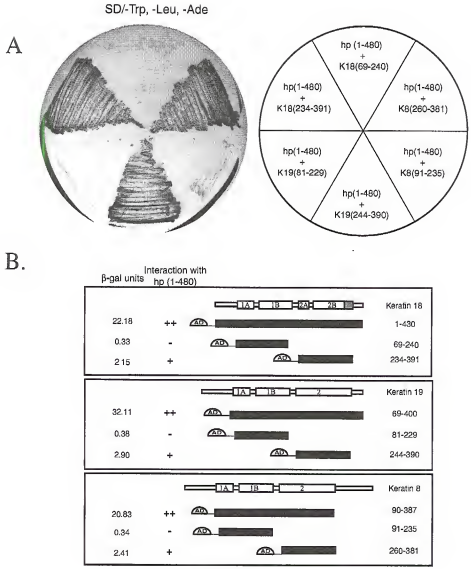


Figure 3.1 Two-hybrid analyses demonstrated that the coil 2 within the rod domain of K18/K8/K19 contained sufficient sequence to bind to the amino terminal domain of pinin hp(1-480). Human pinin (residues 1-480) fused to GAL4- BD and one of the keratin constructs fused to GAL4-AD were cotransformed into the yeast host strain PJ69-4A. Transformants were subjected to -HIS, -Ade, and b-galactosidase selection assays. (A) Yeast hp(1-480) containing the coil 2 constructs, K18(234-391), K8(260-388) and K19(244-390), exhibited growth on -Ade selective media SD/-Trp, -Leu, -Ade, while yeast containing the coil 1 constructs, K18(69-240), K8(91-235), K19(81-229), exhibited no growth. (B) β-galactosidase activity (β-gal units) obtained from quantitative β-gal assay of each transformant confirmed the results from -Ade selection assay that, the coil 2 domain and hp (1-480) interacted with each other to activated the LacZ gene, while no interaction occurred between the coil 1 domain of K18/K8/K19 and hp (1-480).

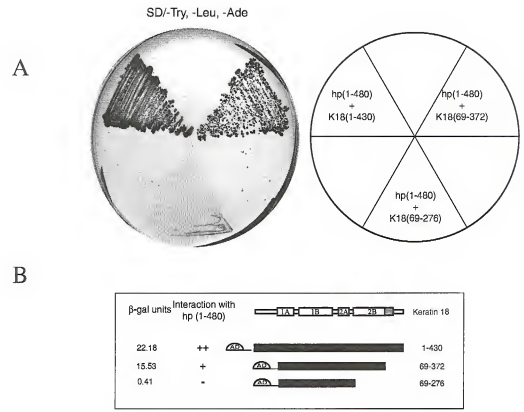
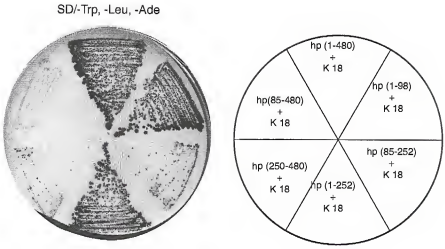


Figure 3.2 Two-hybrid analyses defined the 2B domain of keratin 18 interacting with pinin amino portion 1-480. Either the 2B consensus motif deletion construct K18(69-372) or the 2B domain deletion construct K18(69-276) was cotransformed into yeast PJ69-4A with hp(1-480). The cotransformants were selected on -HIS, -Ade media, and subjected to β-gal assay. (A) Yeast containing K18 (69-372) as well as yeast containing full-length K18 exhibited growth on SD/-Trp, -Leu, -Ade media, while the yeast containing K18 (69-276) exhibited no growth. (B) β-gal assays indicated that K18(69-372) is able to bind to hp (1-480), while K18(69-276) exhibited no binding to hp (1-480).

A.



B.

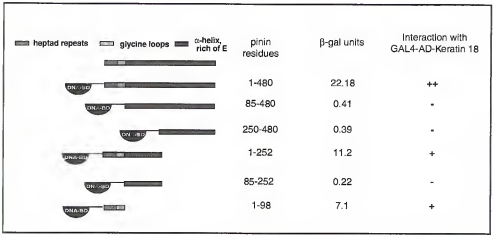


Figure 3.3 Two-hybrid analyses mapped the site in pinin for interacting with keratin 18. Human pinin constructs were cotransformed with K18 into yeast PJ69-4A. As indicated by (A) growth on selective media SD/-Trp,-leu,-Ade. (B) β-gal activity. Pinin fragment hp(1-98), which contains the predicted heptad repeat and glycine loop domains, is sufficient for the interaction of pinin with keratin 18.

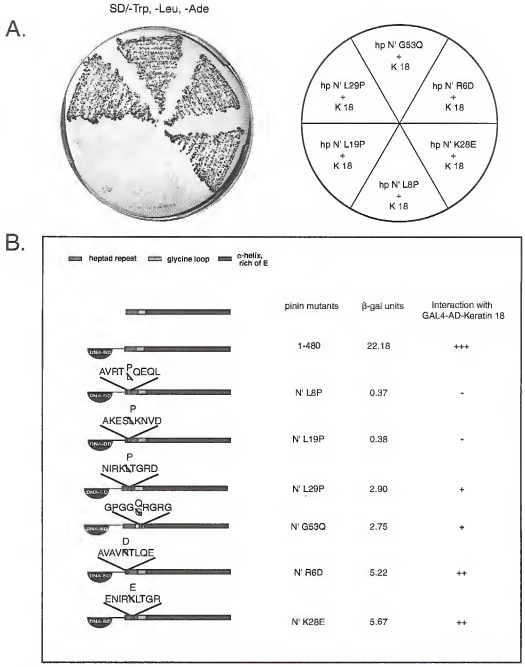


Figure 3.4 Two-hybrid analyses identified the essential residues within hp (1-98) for the interaction between pinin and keratin 18. GAL4-AD-K18 and GAL4-BD-hp N' mutant construct were cotransformed into yeast. (A) N'L8P and N'L19P resulted in no growth on -Ade media, while N'L29P, N'G53Q, N'R6D and N' K28E retained the ability to grow. (B) β-gal assays results indicated no binding between N'L8P/N'L19P and hp(1-480), while N'L29P, N'G53Q, N'R6D, and N' K28E remained to interact with hp(1-480).

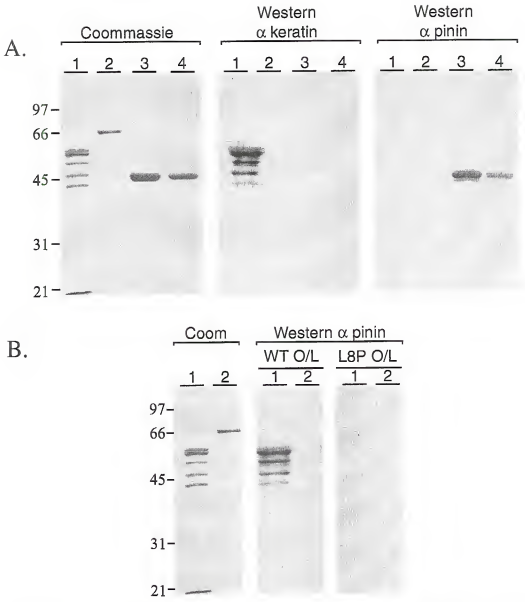


Figure 3.5 In vitro overlay binding assays confirmed the interaction between pinin amino end domain and keratins. (A) SDS-PAGE stained with coommassie blue demonstrated the proteins utlized in the overlay binding assay. Lane 1: purified MDCK keratins; lane 2: BSA; lane 3: pinin GST fusion protein GST-cp(1-165); and lane 4: mutant pinin GST fusion protein GST-cp(1-165) L8P. Keratins were confirmed by western blot probed with anti-keratin antibody (A, lane 1). Both wild type and mutant pinin GST-fusion proteins were recognized by anti-pinin antibody UF 215(A, lane 3 and lane 4). (B) Purified keratins (lane 1) and BSA (lane 2) were overlayed with either wild type GST-cp(1-165) (WT O/L) or mutant GST-cp(1-165) L8P (L8P O/P). Binding of any of these proteins to keratins was detected by western blot using anti-pinin antibody UF215. Wild type GST-cp(1-165) did bind to keratins and was recognized by UF 215, while mutant GST-cp(1-165) L8P did not bind to keratins.

Human pinin residues	PCR primer sets
1-480	GCA CAT ATG ATG GCG GTC GCC GTG AGA ACT
	GCG CGT CGA CTG AGC CTG AGG TTG AGC CAC
85-480	CGG CAT ATG CTG GGC GGG GAG CGT CG
	GCG CGT CGA CTG AGC CTG AGG TTG AGC CAC
250-480	GCG CAT ATG GCT ACC CAA AAA CTA ATA GAA
	GCG CGT CGA CTG AGC CTG AGG TTG AGC CAC
1-252	GCA CAT ATG ATG GCG GTC GCC GTG AGA ACT
	GAG GCG TCG ACG GGT AGC TGG ACA CAT TCT
85-252	GCG CAT ATG GCT ACC CAA AAA CTA ATA GAA
	GAG GCG TCG ACG GGT AGC TGG ACA CAT TCT
1-98	GCA CAT ATG ATG GCG GTC GCC GTG AGA ACT
	GTG CTG TCG ACC CTG GCG TGA TTC TCT TCT

Table 3.1 PCR primer sets for generating the truncated constructs of pinin amino portion domains.

Protein domain	residues	PCR primer sets
K18 coil 1	69-240	GCG ACT CGA GGT CTG GCA GGA ATG GGA GG
		CGC GAA TTC GGG GGC ATC TAC CTC CAC
K18 coil 2	234-391	CGC ACT CGA GAG GTA GAT GCC CCC AAA TC
		GCG GAA TTC ATT AAA GTC CTC GCC ATC TTC
K19 coil 1	81-229	GCG ACT CGA GTA ACC ATG CAG AAC CTC AAC G
		GCG GAA TTC TCC CAC TTG GCC CCT CAG C
K19 coil 2	244-390	CCG TCT CGA GTC GCC AAG ATC CTG AGT GAC
		CGC GAA TTC GTA GTG ATC TTC CTG TCC CT
K8 coil 1	91-235	GCG ACT CGA GAG AAG GAG CAG ATC AAG ACC
		GCT GAA TTC AGC TCC CGG ATC TCC TCT TCA
K8 coil 2	260-381	GCG ACT CGA GCT GAG GTC AAG GCA CAG TA
		GCA GAA TTC CTT GAC GTT CAT CAG CTC CTG
K18	69-372	GCG ACT CGA GGT CTG GCA GGA ATG GGA GG
		CGC GAA TTC CTT GAC CTT GAT GTT CAG CAG
K18	69-276	GCG ACT CGA GGT CTG GCA GGA ATG GGA GG
		CGC GAA TTC CTC AAT CTG CTG AGA CCA GTA

Table 3.2 PCR primer sets for generating truncated K18, K8, and K19 constructs.

pinin point mutant	PCR primer sets
N' L8P	GTC GCC GTG AGA ACT CCG CAG GAA CAG CTG GAA AAG G
	CCT TTT CCA GCT GTT CCT GCG GAG TTC TCA CGG CGA C
N' G53Q	CTG GTG GAG GTA GAG AAC GTG GTA GTT TAT TAC
	GTA ATA AAC TAC CAC GTT CTC TAC CTC CAC CAG
N' L19P	GAA AAG GCC AAA GAG AGT CCT AAG AAC GTG GAT GAG
	CTC ATC CAC GTT CTT AGG ACT CTC TTT GGC CTT TTC
N' L29P	GAA CAT TCG CAA GCC CAC CGG GCG GGA TC
	GAT CCC GCC CGG TGG GCT TGC GAA TGT TC
N' R6D	GCG GTC GCC GTG AAC ACT TTG CAG GAA CAG CTG
	CTG TTC CTG CAA AGT GTT CAC GGC GAC CGC CAT
N' K28E	GAT GAG AAC ATT CGC CAG CTC ACC GGG CGG GAT C
	GAT CCC GCC CGG TGA GCT GGC AAT GTT CTC ATC

Table 3.3 PCR primer sets for the site-directed mutagenesis of pinin amino portion domains.

CHAPTER 4
IDENTIFICATION OF A SUBSET OF RS DOMAIN CONTAINING PROTEINS INTERACTING WITH PININ AND CHARACTERIZATION OF THE RS CONTAINING PROTEIN BINDING DOMAIN IN PININ

Introduction

Pinin was initially identified as a desmosome-IF associated protein and was suggested to be involved in cell-cell adhesion organization and adhesion-cytoskeleton stabilization (Ouyang and Sugrue, 1992; Ouyang and Sugrue, 1996). On the other hand, pinin was also observed present in non-epithelial cells (Ouyang and Sugrue, 1996) and was localized in the nucleus of some cultured cell lines as well as in various tissues (Brandner et al., 1997; Brandner et al., 1998; Ouyang, 1999). It was suggested that pinin may play roles other than involving in cell adhesion-cytoskeleton organization and/or stabilization.

Pinin has been localized to nuclear sub-structures called interchromatin granule clusters as well as throughout the nucleoplasm (Brandner et al., 1997; Brandner et al., 1998; Ouyang, 1999). Little is known about the nature of pinin in the nucleus except that nuclear fractionation analyses detected pinin's presence in the fractions containing splicing factors SF3a, SF3b and 17S U2 snRNP (containing U2 snRNP and SF3a/b) (Brandner et al., 1998). Very likely, pinin play a role in splicing related activities. However, questions pertaining to how pinin integrates to the particular substructure of the nucleus and what role pinin plays there remain to be addressed.

Virtually, in addition to their diffuse distribution throughout the nucleoplasm, all proteins involving in pre-mRNA splicing are enriched in numerous nuclear compartments, such as speckled domains or coiled bodies. Speckled domains and coiled bodies are discernible based on the number of them in the nucleus and the protein components within the substructures. In a typical mammalian cell, there are 20-50 speckled domains and 1-5 coiled bodies. Both of these two structures contain snRNPs, however, speckled domains are enriched in SR proteins while coiled bodies can be marked by a constitutive protein p80-coilin (Lamond and Earnshaw, 1999; Spector, 1993).

Speckled domains have been distinguished as two types of structures by electron microscopy: interchromatin granule clusters (IGCs) and perichromatin fibrils (PFs) (Krause et al., 1994; Spector, 1993). In situ hybridization studies and nucleotide incorporation studies have placed actively transcribed genes outside and at the periphery of IGCs (Hendzel et al., 1998; Misteli et al., 1997; Wansink et al., 1993; Zhang et al., 1994). Upon activation of transcription, pre-mRNA splicing factors were recruited from the IGCs to PFs in a phosphorylation-dependent manner (Misteli, 1999; Misteli et al., 1998; Misteli et al., 1997; Misteli and Spector, 1999). Recently, C-terminal domain of the large subunit of RNA polymerase II was shown to play a role in this dynamic translocation (Misteli and Spector, 1999). Therefore, IGCs are considered as the sites of storage and/or assembly of splicing factors, while PFs are believed to be the sites of active transcription and splicing.

The family of SR proteins is one prominent component of nuclear speckled domains (Valcarcel and Green, 1996). SR proteins have a modular structure consisting of one or two RNA-binding domains (RBD) at the amino terminus and an arginine-serine-rich (RS) region at the carboxyl end of the molecule. SR proteins recruit other splicing factors during spliceosome assembly through protein-RNA interactions via the RBD or through the protein-protein interactions via RS domain. Moreover, SR proteins bind to specific RNA splicing enhancer or exonic splicing enhancers (ESE) and play central roles in both constitutive splicing and regulated alternative splicing (Graveley and Maniatis, 1998; Hertel and Maniatis, 1998; Horowitz and Krainer, 1994; Schaal and Maniatis, 1999; Valcarcel and Green, 1996). At least nine SR proteins have been characterized in mammals, including SRp20 (Tripodis et al., 1998), SRp30a/ASF/SF2 (Krainer et al., 1991), SRp30b/SC35 (Fu and Maniatis, 1992), SRp30c (Screaton et al., 1995), 9G8 (Popielarz et al., 1995), SRp40 (Screaton et al., 1995), SRp46 (Soret et al., 1998), SRp55 (Screaton et al., 1995), and SRp75 (Zahler et al., 1993b). These proteins can individually restore the splicing activity of otherwise splicing deficient Hela cell nuclear extract S100 (Soret et al., 1998; Valcarcel and Green, 1996; Zahler et al., 1993b). In addition, splicing coactivator SRm160 (Blencowe et al., 2000; Blencowe et al., 1998; Eldridge et al., 1999) have been shown to complement to SR proteins, stimulate the splicing activities. Although, structural and functional similarities among SR proteins suggest that they play redundant roles in pre-mRNA splicing, the high degree conservation of individual SR protein from different species and the less homology among members of the SR protein family from the same species indicated that each SR protein has unique function *in vivo* (Valcarcel and Green, 1996; Zahler et al., 1993a).

In an effort to identify proteins interacting with pinin, we have performed a two-hybrid screening using either the amino end domain or the carboxyl terminal domain of pinin as bait. The carboxyl terminal bait (residues 470-717) identified a group of SR proteins or proteins containing RS dipeptide/tetrapeptides domain, including SRp75, SRm300 and a hypothetical SR protein. Sequence analyses revealed an interesting fact that the RS domain within the SR proteins was most likely the binding site of pinin bait except that pinin might have one additional binding site within protein SRm300. Further truncation analyses on pinin elucidated that the sufficient sequence of pinin for interacting with different RS domain-containing proteins varies. This identification of the nuclear proteins interacting with pinin is consistent with previous observation of pinin locating in the nucleus. Furthermore, our data revealed the possible protein relationship of pinin in the specific protein complex (pre-mRNA splicing complex). This, as an innovative work, will confer directions for future studies and will greatly benefit our understanding of pinin functions in the nucleus.

Materials and Methods

Yeast strain and media. Please see chapter two.

Bait construct and two-hybrid screening. The DNA fragment of pinin residues 470-717 was obtained by PCR and cloned in-frame into the GAL4 DNA binding domain (GAL4BD; bait) vector pAS2-1 (Clontech, Matchmaker II sysytem). The GAL4BD-pinin vector was cotransformed with a Clontech Matchmaker cDNA library into the yeast strain PJ69-4A using the yeast transformation method of Gietz *et al* (Gietz et al., 1997).

The library consisted of human fetal kidney cDNA fused to the activation domain of GAL4 (GAL4AD, prey) in the pGAD 10 vector (Clontech).

Approximately 10^6 transformants were first selected on -HIS media. Then the yeast colonies growing on -HIS media were replicated to and selected on -Ade media. Positive colonies from -Ade selection were subjected to liquid culture ONPG β-galactosidase assay according to manufacturers' procedure (Clontech). A well-characterized interaction between p53 and SV40 large T-antigen was used as a positive control in β-gal assays. Baseline level of β-gal activity was determined from negative control yeast that had been cotransformed with GAL4-BD-pinin (470-717) and GAL4-AD. Each value of β-gal units was decided by an average of enzyme activity of 3 independent positive colonies. The "prey" plasmids were recovered from triple positive (HIS, Ade, and LacZ) clones and co-transformed with the control heterologous baits, p53, lamin C, pinin (1-480), and GAL4-BD into the yeast host. In addition, the "prey" plasmid was also transformed by itself into the yeast host to test for possible false-positives. Putative positive clones that were selected from -HIS, -Ade and β-gal selection assays and exhibited no interaction in negative controls were further subjected to sequencing analyses.

To examine the ability of pinin truncations to interact with SRp75 or SRm300, or the hypothetical SRK protein, the GAL4BD vector containing the individual pinin truncations were co-transformed with prey plasmids that containing partial sequence of SRp75 or SRm300 or SRK into PJ69-4A yeast cells. Triple selections described above were applied to the transformants.

Generation of truncations constructs of pinin carboxyl terminal domain. Truncated cDNA of pinin were generated by PCR using the primer sets listed in Table IV. PCR products of human pinin were fused in frame to the GAL4BD in the vector pAS2-1 at Nde I/Sal I sites. All constructs were verified by sequencing.

Results

SRp75, SRm300, and a hypothetical SR protein were identified to interact with pinin carboxyl terminal domain in a two-hybrid screen. A two-hybrid screen using the carboxyl terminal domain of pinin (residues 470-717) as bait identified 22 positive clones. Extensive control analyses on these clones were conducted to ensure that the activation of the reporter genes in the two-hybrid assay was indeed due to the interaction between the bait and the prey (see Materials and Methods and also see table 2.2). Sequence analyses revealed several clones grouped as RS domain containing proteins, including SRp75 (clone C-54-1), SRm300 (clone C-25-10 and C-34-3), and a hypothetical SR protein (clone C-15-8).

Clone C-54-1 encoded residues 117-494 of an arginine-serine-rich splicing factor SRp75 (494 aa). C-25-10 encoded residues 129-712 and C-34-3 encoded residues 1-200 of a subunit of a splicing coactivator SRm300 (2296 aa). C-15-8 encoded partial sequence of an uncharacterized hypothetical SR protein (17-299 of >299aa), and this SR protein sequence was also found in the human EST database, indicating it is a truly expressed protein. Sequences of aforementioned four clones were compared with corresponding sequences from the Genbank database. Interestingly, clone C-54-1 contained the entire RS domain of SRp75 (~315 aa long) while several amino acids of the

upstream RNA recognition motif homolog (RRMH) were included in the clone (Fig. 4.1). SRm300 has remarkable high content of serine (S), arginine (R), and proline (P). It contains numerous of RS dipeptides/tetrapeptides presenting as two RS clusters and it also contains two polyserine domains with unprecedented length (25 and 41 residues, respectively). No RNA binding motif was found in the SRm300 sequence. However, the N-terminal 159 amino acids of SRm300 show significant similarity to the N-terminal region of a hypothetical protein conserved from yeast to human (Blencowe et al., 2000). Clone C-25-10 encoded the major region (~230 aa) of the first RS cluster of SRm300. Interestingly, clone C-34-3 encoded the N-terminal region of SRm300 including aforementioned conserved N-terminal domain but almost contained no RS dipeptides. It seemed that pinin may recognize two binding sites within SRm300 and these sites are likely adjacent to each other (Fig. 4.2). The hypothetical SR protein as referred to by the database had only an incomplete sequence available and the start codon of this protein was missing. No RNA binding motif was found, although it is possible that an upstream RBD exists in the uncovered sequence. Clone C-15-8 encoded the region of the hypothetical SR protein that contains multiple RS dipeptides/tetrapeptides (~260 aa) (Fig. 4.3). In summary, pinin was capable of directly binding to a subset of proteins that contain a long stretch of RS dipeptides/tetrapeptides.

Different domains of pinin bind to individual RS domain containing protein. The carboxyl terminal bait used in the two-hybrid screen was pinin residues 470-717, which include the "QLQP" repeat domain, the poly-serine domain predominantly consisted of serine residues with several RS dipepetides/tetrapeptides sparsely distributed, and a highly positively charged DRK repeat domain also containing a few RS

dipeptides/tetrapeptides (Ouyang and Sugrue, 1996). Five truncation constructs of pinin were generated and employed in two-hybrid analyses to define the sufficient sequence of pinin for interacting with each of the RS domain- containing proteins. As judged by growth on selective media and by quantitative β-gal assays, poly-serine domain and the DRK repeat domain (residues 559-717) together were sufficient for interacting with either clone C-54-1 (SRp75 residues 117-494) (Fig. 4.4) or C-34-3 (SRm 300 residues 1-200) (Fig. 4.6). Residues 559-642 containing only the poly-serine domain were sufficient to interact with C-15-8 (The hypothetical SR protein, residues 17-299), and residues 470-642 and 559-717 that contain poly-serine domain as well as additional flanking domain were capable of interacting with C-15-8 (Fig. 4.7). However, clone C-25-10 (SRm 300 residues 129-712) needed both the poly-serine domain and the "QLQP" repeat domain to interact with pinin (Fig. 4.5).

It is noticed that pinin residues 470-717 may bind to SRp75 in very high affinity as indicated by the β-gal unit from the quantitative β-gal assays and by the vigorous growth on -Ade selective media (Fig. 4.4). In addition, pinin residues 559-717 that are lack of the "QLQP" domain can bind to SRm300 stronger than the longer construct pinin residue 470-717.

Discussion

We, in this study, identified three RS dipeptides containing proteins including SRp75, SRm300, and a hypothetical SR protein, that directly interacting with pinin in a two-hybrid screening. Sequence analyses indicated the possible domains within these RS domain-containing proteins that mediate the interaction with pinin. Truncation analyses

further revealed the possible domain within pinin that may be sufficient for the interaction. These results are not only consistent with the previously observed nuclear speckle-like immunostaining of pinin, but further uncovered the possible molecular relationship of pinin with the spliceosome complex and/or speckled substructure. Our data strongly suggested that pinin may be involved in RS domain containing proteins, specifically SRp75 and SRm300, related cellular activities, and thus paved the path for future studies on the function of pinin in the nucleus.

Full-length cDNA of both pinin and SRp75 have been transiently transfected into HEK 293 cells. Immunofluorescence analyses of the exogenously expressed proteins showed that pinin and SRp75 were co-localizing together as numerous foci in the nucleus (This work was done by Matt Simmons, a graduate student in the lab). This, complementary to the yeast two-hybrid assays, demonstrated that pinin and SRp75 are able to bind to each other in the cultured mammalian cells.

Studies in the lab have employed an anti-pinin antibody UF 215 and mAb 104 (a monoclonal antibody specifically recognizes a phospho-epitope in the RS domain of SR proteins) to analyze the endogenous localization of pinin and SR proteins in MDCK cells. A parallel study was also performed using Y12 (an antibody recognizing core proteins of snRNP, Sm proteins) instead of mAb 104 in a similar co-localization analysis. Interestingly, Y12 immune antigens seemed to be localized more adjacent to pinin, while mAb 104 staining were relatively distant (This work was done by Matt Simmons). It is not yet clear why pinin preferred Sm proteins to phosphorylated SR proteins at the given moment. However, as it is well known, the distribution of SR protein as well as other splicing factors in the nucleus is very dynamic. Both phosphorylation status (Mermoud

et al., 1994; Misteli, 1999; Misteli et al., 1998) and transcriptional activities (Misteli and Spector, 1999) have been reported to affect the translocation of splicing factors including Sm proteins and SR proteins, in turn affect their distributions within the nucleus. Investigation on whether or not pinin co-localizes with active transcription sites (could be detected by [^{3}H] Br-UTP labeling) is expected to shed light on understanding of these morphological observations as well as on further directions pertaining to the function of pinin in the nucleus.

The sequence analyses revealed an interesting possibility that pinin may bind to the RS domain of the three identified proteins. RS domain has been long known to function primarily in protein-protein interactions with other SR proteins via phosphorylated RS domain (Lamond and Earnshaw, 1999; Valcarcel and Green, 1996). RS domain may also participate in the targeting of SR proteins to speckles and may be important for the integrity of these subnuclear structures (Caceres et al., 1997; Li and Bingham, 1991; Misteli et al., 1998). Pinin is not considered as a typical SR protein since it does not contain recognized RNA binding motif. However, the carboxyl terminal domain of pinin possesses a few RS dipeptides within the polyserine domain and the DRK repeat domain (Ouyang and Sugrue, 1996). Possibly, pinin possesses a RS domain recognition site that specifically interacts with a subset of RS domain containing proteins. Further truncation analyses on those RS domain- containing proteins and site-directed mutageneses targeting on residues of pinin will test this hypothesis.

It was noticed that pinin preferred to interact with proteins with long RS domain/or large number of RS dipeptides in the two-hybrid screen. SRp75 has an unusual long RS domain (315 aa) comparing to RS domain of the other 8 characterized

SR proteins (30-100aa). SRm 300 possesses two RS domains and the one interacting with pinin is ~230 aa long. The hypothetical SR protein also has a RS domain in length of ~260 aa. One possibility could be other SR proteins cDNA were missed in the human fetal kidney library that we screened. However, the fact that SRm160, as a protein contains extensively distributed RS dipeptides and has been shown to be ubiquitously expressed in tissues, was not identified by pinin in the two-hybrid screen strongly argues aforementioned possibility. Nevertheless, we can not rule out the possibility of SRm160 interacting with pinin at this time. SR proteins have been demonstrated to bind to ESE to promote the splicing activities. Graveley et al. provided quantitative data suggesting that the splicing activity of the bound SR proteins on ESE is directly proportional to the number of RS tetrapeptides within the RS domain (Graveley et al., 1998). That pinin specifically bind to long RS domain containing proteins or SR proteins with specific RS domain composition to the active splicing site to enhance the splicing activity is an interesting speculation waiting for experimental attack.

It is appealing to identify SRp75 and SRm300 interacting with pinin. Both SRp75 and SRm300 have been localized at the interchromatin granule clusters in the nucleus (Blencowe et al., 2000; Eldridge et al., 1999; Zahler et al., 1993b), and one immunoprecipitation assay has found these two proteins co-precipitated together with SRm160 and SRp40 from the nuclear extract (Blencowe et al., 1998). SRp75 is so far the largest SR protein characterized. Comparing to others, it has a long RS domain and has been reported to be involved in constitutive and alternative splicing by associating with splicing complex and with RNAs (Zahler et al., 1993b). SRm300 was originally identified as a subunit of a pre-mRNA splicing coactivator SRm160/300 (Blencowe et al.,

1998). Although SRm300 and SRm160 have been seen coincident in speckled domains in interphase cells, they have different distributions during mitosis (Blencowe et al., 2000). SRm160 alone can complement the splicing deficiency of Hela cell S100 extract with the addition of limiting amount of SR family proteins, and when SRm160 were immunodepleted from nuclear extracts, PIP85A pre-mRNA splicing was prevented (Blencowe, 1998, 2000). In addition, SRm160 was required for a GAA-repeat exonic splicing enhancer (ESE) to promote the splicing of a pre-mRNA containing a weak 3' splice site (Eldridge et al., 1999). SRm300, on the other hand, failed to show the similar involvement in splicing activity as SRm160. However, during the steps of the splicing reaction on different pre-mRNA substrate, SRm300 is stably associated with SRm160 and with splicing complex (Blencowe et al., 2000). Additionally, The presence of extensive RS domain in SRm300 molecule strongly suggested that it is capable of interacting simultaneously with many factors, including SRm160 and SR proteins (Blencowe et al., 2000). Thus, within the splicing complex, SRm160 may be more directly involved in the splicing activity, while SRm300 more likely play a role different but related. It was proposed that SRm160/300 function as a coactivator of ESE-dependent splicing by bridging between basal snRNP components of the spliceosome and SR protein "activators" bound to an ESE (Fig. 4.8) (Eldridge et al., 1999). Pinin probably fits into this model by interacting with either or both SRp75 and SRm300.

It is intriguing that different pinin residue stretches are sufficient for interacting with the two SRm300 clones and with SRp75 clone. SRp75 can bind to pinin at residues 559-717, while SRm300 can bind to pinin at both residues 559-717 and 470-642 via different but adjacent domains. It is possible that pinin binds to SRm300 in an anti-

parallel way that the carboxyl terminus of pinin binds to the amino end of SRm300 while the rest of SRm300 or pinin interacting with other complex components (Fig. 4.9). Although the poly-serine domain and the DRK repeats domain together are necessary for both SRp75 clone and one of the SRm300 clone binding to pinin. The possibility of pinin associating with both SRp75 and SRm300 simultaneously is not excluded.

In summary, we have identified SRp75, SRm 300, and a hypothetical SR protein directly interacting with the carboxyl terminal domain of pinin. This work is important for our understanding of the function of pinin in the nucleus, and will greatly contribute to our future studies.

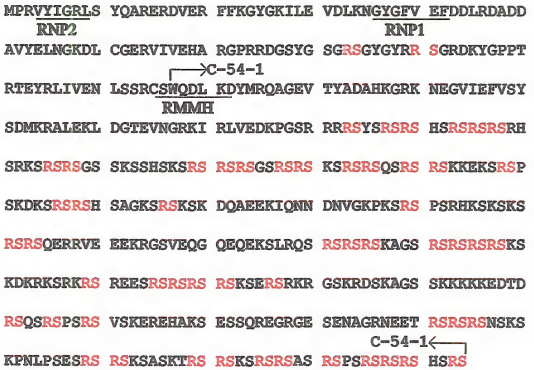


Figure 4.1 Clone C-54-1 matches to residues 117-494 of SRp75 coding sequence. The full length SRp75 protein sequence is displayed. The up-stream RNA binding motifs are underlined and the RS dipeptides/tetrapeptides are printed in red. The sequence matched by Clone C-54-1 is indicated by arrow.

Figure 4.2 Clone C-25-10 and C-34-3 matches to SRm300 at residues 129-712 and 1-200, respectively. Due to the limited space, only part of the SRm300 protein sequenceis displayed. RS dipeptides/tetrapeptides are printed in red while the sequence matched by C-25-10 and C-34-3 is indicated by arrows.

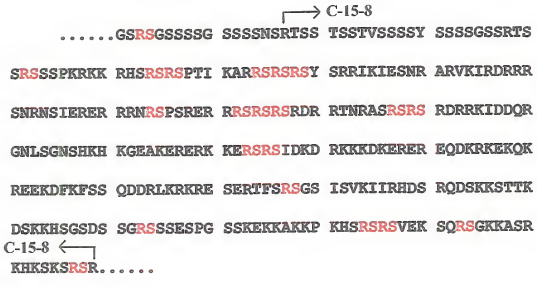


Figure 4.3 Clone C-15-8 matches to part of a hypothetical protein SRK. The available SRK protein sequence is displayed with the RS dipeptides/tetrapeptides printed in red. The sequence matched by C-15-8 is indicated by arrows.

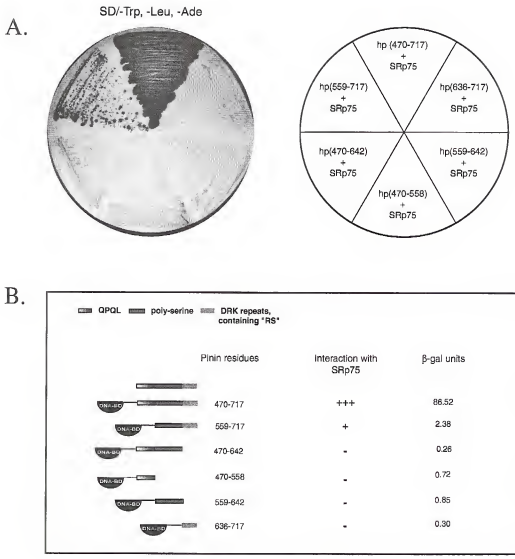
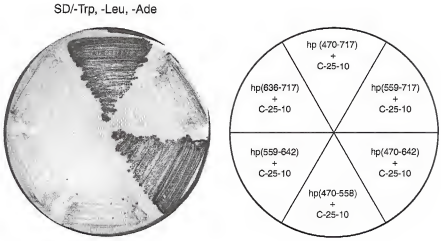


Figure 4.4 Two-hybrid analyses defined the sufficient sequence for pinin interacting with clone C-54-1 (Residues 117-494 of SRp75). Truncation constructs of pinin were generated as indicated that each construct contains various region of pinin. These constructs were individually co-transformed with clone C-54-1 in to the yeast PJ69-4A and subjected to -HIS, -Ade, and β-gal assays selections. As indicated by the growth on -Ade media (A) and by the enzyme activity unit of β-galactosidase (B), residues 559-717 is necessary and sufficient for pinin binding to clone C-54-1 in the two-hybrid assay.

A.



B.

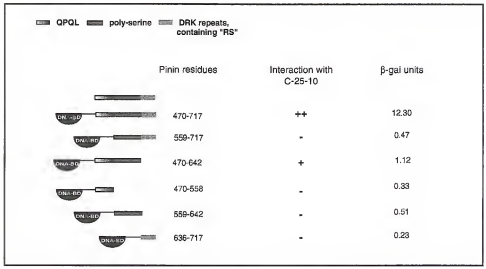
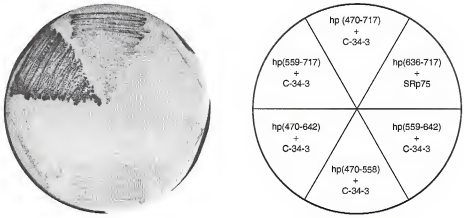


Figure 4.5 Two-hybrid analyses defined the sufficient sequence of pinin for binding to Clone C-25-10 (residues 129-712 of SRm300). The same set of pinin truncation constructs as in Fig 4.4 was co-transformed with C-25-10 and subjected to -HIS, -Ade, and β-gal assays. The growth on -Ade media (A) and the β-gal unit (B) indicated that pinin residues 470-642 are sufficient for pinin interacting with C-25-10.

A.



B.

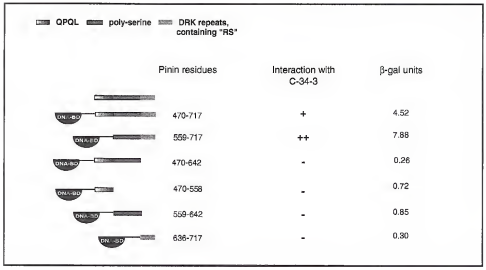
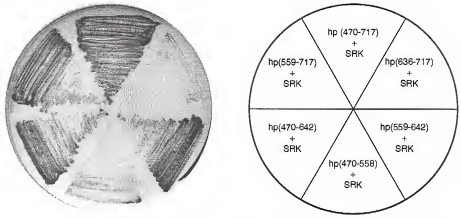


Figure 4.6 Two-hybrid analyses defined the sufficient sequence for pinin binding to Clone C-34-3 (residues 1-200 of SRm300). The same set of pinin truncation constructs as in Fig. 4.4 was co-transformed with C-34-3 and subjected to -HIS, -Ade, and b-gal assays. The growth on -Ade media (A) and the b-gal unit (B) indicated that pinin residues 559-717 are sufficient for pinin interacting with C-34-3. It is noted that the construct 559-717 resulted in an increase of the b-gal unit, indicating the interaction between residues 559-717 and clone C-34-3 may be stronger than the interaction between residues 470-717 and clone C-34-3.

A.



B.

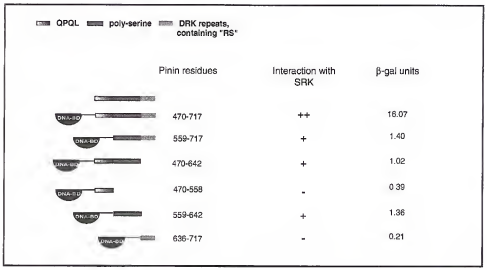


Figure 4.7 Two-hybrid analyses defined the sufficient sequence for pinin interacting with clone C-15-8 (residues 17-299 of SRK protein). The same set of constructs as in Fig. 4.4 was contransformed with clone C-15-8 and subjected to –HIS, -Ade, and β-gal assays. The growth on -Ade media (A) and the β-gal unit (B) indicated that pinin residues 559-642 are sufficient for pinin interacting with C-15-8. Residues 559-717 and 470-642, which both have flanking sequence in addition to residues 559-642, are also capable of binding to C-15-8.

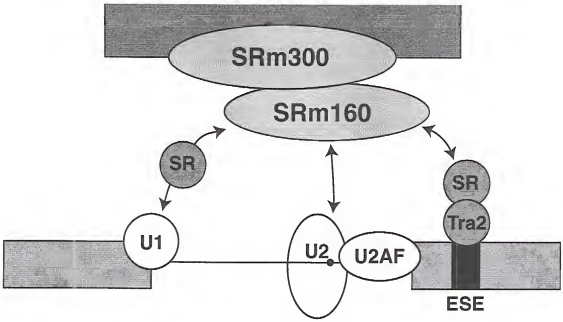


Figure 4.8 A model of SRm160/300 involving in spliceosome (Eldridge et al., 1999).

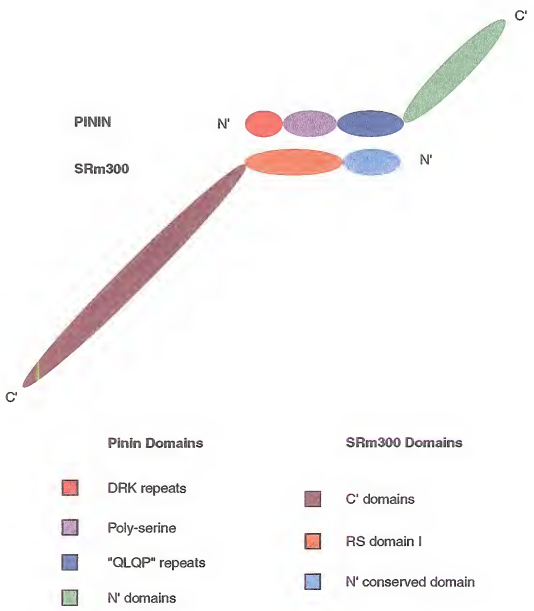


Figure 4.9 A model of anti-parallal interaction between pinin and SRm300.

human pinin residues	PCR primer sets
470-717	GCG CAT ATG GCT ACC CAA AAA CTA ATA GAA CGC CGT CGA CAT TAA CGC CTT TTG TCT TTC CTG T
559-717	GCG CAT ATG GCT ACC CAA AAA CTA ATA GAACGC CGC CGT CGA CAT TAA CGC CTT TTG TCT TTC CTG T
470-642	GCA CAT ATG GAA TCT GAG CCC CAA CCT GAG GTG CTG TCG ACG CTT TCT ATC TCT ATT ATG TCC C
470-558	GCG CAT ATG GCT ACC CAA AAA CTA ATA GAA GAG GCG TCG ACG GGT AGC TGG ACA CAT TCT
559-642	GCG CAT ATG GCT ACC CAA AAA CTA ATA GAACGC GTG CTG TCG ACG CTT TCT ATC TCT ATT ATG TCC C
636-717	CGA CAT ATG GGC CGG GGA CAT AAT AGA GA CGC CGT CGA CAT TAA CGC CTT TTG TCT TTC CTG T

Table 4. 1 PCR primer sets for generating the truncated constructs of pinin C-terminal domain

CHAPTER 5
SUMMARY AND PERSPECTIVES

This study has focused on identification of proteins that directly interact with pinin and characterization of domains of pinin that specifically mediate the binding to these different partners. Intriguingly, two major groups of proteins and several others have been revealed to be capable of binding to pinin. One group are nuclear proteins related to the pre-mRNA splicing and they can bind to the C-terminal domain of pinin. The other group are either junction-cytoskeleton complex proteins or proteins involving in protein transport to the nucleus, and they can bind to the N-terminal domain of pinin. These results, coupled with the previous observations, present a plausible but complicated scenario of the cellular activities and molecular relationships involved by pinin, lending support to the dual location theory of pinin as well as suggesting new hypotheses.

Pinin may directly bind to keratins and potential desmosomal proteins to carry out its role at the sites of desmosome-IF complex. The direct interaction between pinin and keratins has been characterized in chapter three. This data provided significant advance to the understanding of the relationship between pinin and IFs. Meanwhile, the identification of two potential desmosomal proteins (periplakin-like, trichohyalin-like) interacting with pinin leads us towards the hypothesis that pinin may interact with keratin and be landed specifically near or at the desmosome by one or both of the proteins.

Even though a correlation of pinin's location at the desmosome and the enhanced organization of keratin filaments was observed (Ouyang and Sugrue, 1992), no solid evidence has been provided to demonstrate either pinin directly binding to intermediate filaments *in vitro* or the filament-like decoration of pinin to IFs *in vivo*. It is argued that whether pinin indeed binds to IFs *in vivo*. The concern is the coiled-coil hydrophobic surface of keratins has been sealed within the coiled-coil as IFs assemble. If pinin binds to keratin via a coiled-coil interaction, the 2B domain coiled-coil surface of keratin subunit that was available in the two-hybrid system would have already been buried and no longer available any more *in vivo*. In fact, point mutation analyses in the two-hybrid assays exhibited different effect on the interaction resulted from substitutions of residues at the critical sites of putative heptad repeats. Additionally, the deficiency of "trigger" sequence within the amino end of pinin is unfavorable to the formation of coiled-coil structure in pinin (Kammerer et al., 1998; Steinmetz et al., 1998). Therefore, it is likely that pinin may bind to keratins 2B domain via its amino end domain through protein-protein interaction other than forming coiled-coil. The binding of pinin to IFs might resemble to the binding of desmoplakin to K8/K18 filaments that occurs between desmoplakon to the rod domain of K8/K18, which has been illustrated in the two-hybrid assays (Meng et al., 1997)and in transfection analyses (Stappenbeck et al., 1993). Addition, a hemidesmosomal protein BPAG 2 was also found to bind to K18 via the 2B domain (Aho and Uitto, 1999). Possibly, this connection to the rod domain of K8/K18 filaments represents one type of the desmosome/hemidesmosome specific IFs associations in the simple epithelial cells.

In the nucleus, pinin may be involved in spliceosome complex formation via binding to SRm300, SRp75, and other potential SR proteins, playing a role as a connecting protein between SRm160/300 and SR proteins. SRm160/300 has been proposed to function as a coactivator of exonic splicing enhancer (ESE)-dependent splicing by bridging between basal snRNP components of the spliceosome and multiple SR proteins bound to an ESE (Eldrige, 1999). Within this protein complex, multiple snRNPs associate and coordinate to recognize the splicing site of the nascent mRNA. SR proteins, with the ability to bind to RNA via its RNA binding motif and the ability to bind to other SR proteins via its phosphorylated RS domain, may associated with snRNPs and other SR proteins simultaneously. Meanwhile, specific SR proteins bind to the ESE to facilitate the recognition of 3' splicing site. The coordination between snRNPs and SR proteins bound to the pre-mRNA ESE sequence has to be accomplished, but is largely not understood. The association of SRm160/300 with a subset of SR proteins coupled with the coactivator function of SRm160 suggested that SRm160/300 may mediate the connection of the snRNPs and the SR proteins (Fig. 4.8). However, no evidence has been shown on how SRm160/300 associates with snRNPs and with SR proteins. The fact that pinin may directly bind to SRm300 and SRp75 suggests a possibility of pinin mediating the connection between SRm160/300 and SRp75, and in turn to other SR proteins either bound to ESE or to snRNPs.

Pinin may also mediate the connection of interchromatin granule clusters (IGCs) and the putative nuclear matrix core filaments. A stable association of SRm160/300 with the nonchromatin nuclear matrix indicates that they may function in close association with this substructure *in vivo*. The structural and functional nature of nuclear matrix has

not been fully understood, yet. However, it becomes clear that the intranuclear biochemical events such as DNA replication, gene transcription, and post-transcription processing are occurring at specific substructures of the nucleus (Lamond and Earnshaw, 1999; Nickerson et al., 1995; Pederson, 1998). These nuclear substructures reside in the extensive network of matrix core filaments as individual functional unit, allowing for efficient activities by organizing involving proteins. Structural relationship between the core filaments and nuclear substructures such as IGCs remains largely unknown. Several groups have reported the existence of the core filaments in the nucleus (Berezney and Coffey, 1974; Hozak et al., 1995; Jackson and Cook, 1988). Nickerson et al (Nickerson et al., 1997; Wan et al., 1999) have employed a resinless protocol to prepare the nuclear matrix and uncovered the nuclear matrix consisting of a network of intricately structured fibers connected to the nuclear lamina and these fibers are built on an underlying network of branched 10 nm filaments. It is appealing to think that IF protein or IF like proteins would compose the 10 nm nuclear core filaments. There is evidence of IF proteins residing in the nucleus. For instance, lamin A has been seen localizing in the coiled bodies and gems of human erythroleukemia cells (Neri et al., 1999). The Traub and Shoeman group (Wang et al., 1996) demonstrated the ability of vimentin selectively binding to DNA fragments in vitro and Keratin 8, 18 and 19 were cross-linked to nuclear DNA in human breast cancer cells (Spencer et al., 1998). In addition, Keratin 19 was shown to be capable of binding to RNA polymerase II core subunit 11 in a two-hybrid assay (Bruno, 1999). However, so far none of the examined proteins (Nickerson et al., 1997; Wan et al., 1999) have been shown to localize with the nucleus in a pattern of the core filaments, which leaves the nature of the core filaments remain to be determined.

We have identified pinin amino end domain capable of binding to IF protein keratin 18, keratin 8, and keratin 19 (Shi and Sugrue, 2000c). It is tempting to speculate that the component of the filaments would be a member of IF protein family and pinin is able to bind to both the filament protein and SRm300, functions as a linker between the filaments and the IGCs.

The nature of pinin peptide sequence including the amino end heptad repeats, glycine loops, central potential α-helical domain, proline-rich "QLQP" domain and the carboxyl poly-serine doamin with RS motifs, confers pinin the ability to be involved in multiple protein-protein interactions (Degen et al., 1999; Ouyang, 1999; Ouyang and Sugrue, 1996). Data from this study depicted a possibility that pinin may function as a linker connecting structural skeletal filaments and functional protein complexes such as the desmosome at the cell-cell adhesion and the spliceosome in the nucleus. Additionally, with similar strategies applied in chapter three and chapter four, we are mapping the specific protein binding domains within pinin. Preliminary data revealed an intriguing phenomena that polarized location of pinin-binding proteins is correlated with polarized protein-binding domains in pinin molecule: the amino portion domain of pinin binds to proteins at the adhesion junction sites, while the carboxyl portion of pinin binds to proteins in the nucleus. An interesting question arise is, what does the amino domains do in the nucleus and what does the carboxyl domains do at the adhesion junctions? The existence of 10 nm filaments netwok in the nucleus has been observed for years. The mRNA localization at the cell periphery, especially at the sites of tension, has also been demonstrated recently (Bertrand et al., 1998; Chicurel et al., 1998). We tempt to speculate that the carboxyl domain of pinin may bind to potential SR-like proteins that

sequester mRNA near the junctions. While in the nucleus, the IF-binding domain of pinin may interact with potential IF-like proteins to anchor the interchromatin granule clusters to the nuclear matrix (Fig. 5.1).

Junction-cytoskeleton activities and nuclear pre-mRNA splicing or nuclear matrix structure are geographically isolated. It would be simple to assume that these events are biologically unrelated. However, the fact that pinin joins to a list of proteins including plakophilin 1/2/3 (Bonne et al., 1999; Mertens et al., 1996) , protein 4.1 (Lallena and Correas, 1997), plakoglobin (Ben-Ze'ev, 1999; Rubenstein et al., 1997), β-catenin (Miller and Moon, 1997; Simcha et al., 1998), etc., exhibits dual location at the cell-cell junctions as well as in the nucleus, leads to a question pertaining to whether the functions at the dual locations are mutually exclusive or coordinate to couple the junction-nucleus activities. Recently, accumulative evidence has been presented to show that nuclear activities such as transcription can be affected by the alteration of nuclear matrix structure and the nuclear structure alteration could be a consequence of signaling from cell adhesion-cytoskeleton and extracellular matrix (Bissell et al., 1999; Ingber, 1997; Lelievre et al., 1998). It is tempting to speculate that pinin plays a role at the desmosome-IF complex both structurally and functionally. Either up-regulation or down-regulation of pinin will resulted in a signaling cascade or even direct transport of pinin between the nucleus and cytoplasm, in turn to affect the nuclear structure/specific gene expressions and cell-cell adhesions.

Identification of protein partners of pinin allows for tempting speculations on the functions of pinin in the cell. Characterization of domains requisite for the interactions provides a great opportunity for exploring pinin's functions. For instance, to interfere

with pinin's participation in a specific protein complex provides a convenient strategy to manipulate the involvement of pinin in different locations or functions. We are looking forward to advance our understanding on the functions of pinin as well as in cell-cell adhesions.

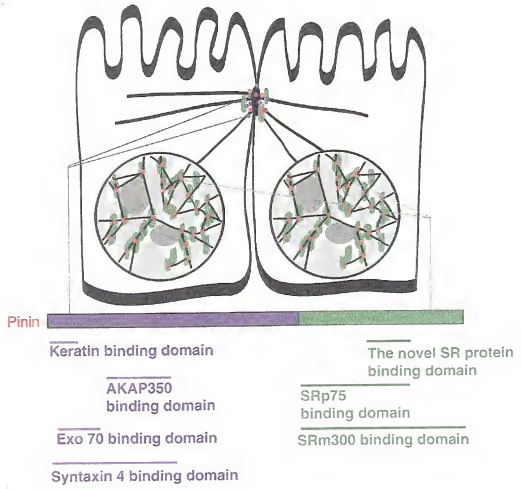
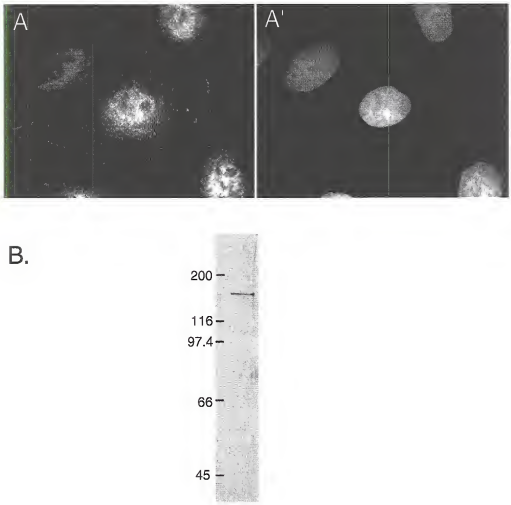
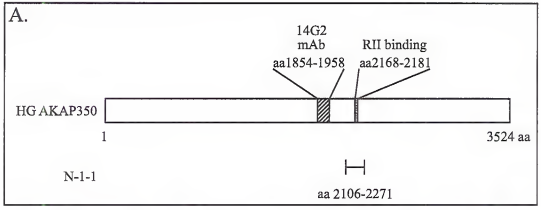
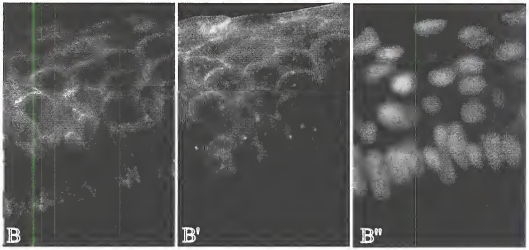


Figure 5.1 A correlation between polarized pinin domains and polarized locations of pinin-binding proteins.



Appendix I. Dual location of pinin in MDCK cells. (A/A') Immunofluorescence demonstrated the dual location of pinin in cultured MDCK cells. Cultured MDCK cells were treated with 0.5% of Triton X-100 for 1 min, then fixed in 2% paraformaldehyde for 10 min. Immunofluorescence was performed with pinin polyclonal antibody UF 215 as primary antibody and rhodamine-conjugated anti-rabbit IGG as secondary antibody. UF 215 antigen was seen at the cell –cell boundary as well as in the nucleus. (B) Western blot confirmed the recognition of pinin by UF 215. Whole cell lysate of MDCK cells was subjected to SDS-PAGE and transferred to nitrocellulose membrane. The blot was incubated with UF 215 as primary antibody and the antigen-antibody interaction was visualized by ECL. The single band detected has the size of ~140 KD which is the known size of pinin. The fact that no cross-reaction occurred in the westernblot suggests UF 215 specifically recognizes pinin.





Appendix II. Pinin and AKAP interact in a two-hybrid analysis and colocalize at the lateral cell boundary in cornea. (A) A two-hybrid screen of a human fetal kidney library using pinin residues 1-480 as bait identified a clone with a 500bp fragment coding for residues 2106-2271 of A-kinase anchoring protein 350 (AKAP350). This 500bp contains the binding site of AKAP350 for PKA regulatory subunit II (RII). (B/B'/B") Co-immunofluorescence using anti-pinin mAb O8L and anti-AKAP350 mAb 14G2 was performed to detect the association of pinin and AKAP350 in vivo. In corneal epithelial cells, pinin (B) and AKAP350 (B') were observed to colocalize at the lateral cell boundary. DAPI staining of the nuclei is shown in B".

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BIOGRAPHICAL SKETCH

Jia Shi was born in Changsha, Hunan, People's Republic of China and attended secondary schools in Changchun, Jilin and in Changsha, Hunan. She went to Wuhan University as an undergraduate where she took cell biology as a major. Following Wuhan University, she enrolled in the Master's program in the Institute of Zoology, Chinese Academy of Sciences, specializing her studies in neuroendocrinology. Her master's thesis entitled "Immunohistochemistry of Dopamine and Norepinephrine in Human Placental Villi" was published in *Chinese Science Bulletin*. In 1994, she joined the Ph.D. program in the department of anatomy & cell biology, University of Florida, the United States, and started her journey on this continent. Over the years, she was working on identification of protein partners of pinin, and had attended several international and national meetings to present her research data. Part of her Ph.D. thesis work, "Dissection of protein linkage between keratins and pinin, a protein with dual location at desmosome-intermediate filament complex and in the nucleus" has been accepted for publication in *Journal of Biological Chemistry*. She plans to join Elizabeth D. Hay's lab in Harvard medical school after she finishs her Ph. D.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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